University of Alberta

The action of antidepressant/antipanic drugs on $GABA_A$ receptor subunit mRNA levels

by

Véronique Anne-Marie Isabelle Tanay ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment
of the requirements for the degree of Doctor of Philosophy

Department of Pharmacology

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Submitted on this day,

Sept 21/98

"L'essentiel est invisible pour les
yeux".... "C'est le temps que tu
as perdu pour ta rose qui fait ta
rose si importante."

"The essential is invisible to the
eyes".... "It is the time spent for
your rose that makes it so
important."

Le Petit Prince
Antoine de Saint-Exupéry

University of Alberta

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled The action of antidepressant/antipanic drugs on GABA_A receptor subunit mRNA levels submitted by Véronique Anne-Marie Isabelle TANAY in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Abstract

Panic disorder is a common mental illness with important economic and social consequences. The neurobiology of this disorder remains unclear, however, since the monoaminergic hypotheses do not account for the wide range of agents that are used either to induce panic in laboratory settings, or to treat this disorder. The monoamine oxidase inhibitor, phenelzine, the tricyclic antidepressant, imipramine, and the benzodiazepine, alprazolam, can all be used to treat panic disorder. Preclinical studies have shown that GABAergic transmission blockade in specific brain regions induces arousal and fear behaviors in laboratory animals, and evidence suggests that $GABA_A$ receptor function may be altered in panic disorder patients. Alprazolam, impramine and phenelzine have been reported to enhance GABAergic transmission by elevating brain GABA levels (phenelzine), increasing GABA release (imipramine), or positively modulating $GABA_A$ receptor function (alprazolam).

$GABA_A$ receptors are responsible for the majority of GABA-mediated neuronal inhibition in the mammalian CNS. Molecular biology has revealed a large $GABA_A$ receptor gene family, but the composition of native $GABA_A$ receptors is not known. Chronic treatment with drugs that affect $GABA_A$ receptor function induces specific changes in $GABA_A$ receptor gene expression. The central hypothesis of this dissertation is that chronic administration of antipanic drugs will commonly alter $GABA_A$ receptor subunit mRNA levels.

Multiprobe solution hybridization was used to determine the steady-state levels of mRNAs that encode $GABA_A$ receptor subunits (α1-6, β1-3, γ1-3) in the brain of rats treated for 3 or 21 days with impramine, phenelzine or alprazolam. As a negative control, buspirone, an anxiolytic devoid of antipanic properties, was included.

The results revealed that, compared to buspirone, the antipanic agents increased α3-, β1- and γ2-subunit mRNA levels in the brainstem, and α6-subunit gene expression in the cerebellum. It is suggested that the alteration in brainstem α3-, β1- and γ2-subunit

mRNA levels after 3 days of alprazolam or 21 days of imipramine or phenelzine administration may correspond to the upregulation of the $\alpha3\beta1\gamma2$ receptor subtype and underlie the onset of antipanic action. Further, it is speculated that the increase in cerebellum $\alpha6$-subunit transcript levels induced by 21 days of drug delivery may correlate with an upregulation of the $\alpha6\beta2/3\delta$ $GABA_A$ receptor subtype in cerebellar granule cells, and might be linked to the relief of anxiety that characterizes the stabilization phase of antipanic drug treatment.

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Abbreviations

ACTH	adrenocorticotropin hormone
AP-1	activator protein-1
APS	ammonium persulfate
CCK	cholecystokinin
CNS	central nervous system
CSF	cerebrospinal fluid
DBI	diazepam-binding inhibitor
DEPC	diethylpyrocarbonate
DMCM	methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate
DMSO	dimethylsulfoxide
DSM	Diagnostic and Statistical Manual for Mental Disorders
DTT	dithiothreitol
GABA-T	GABA transaminase
GAD	glutamic acid decarboxylase
GAT	GABA transporter
LC	locus coeruleus
MAO	monoamine oxidase
MHPG	3-methoxy-4-hydroxyphenylglycol
NTS	nucleus tractus solitarius
PAG	periaqueductal gray
PBS	phosphate-buffered saline
PCI	phenol-chloroform-isoamyl alcohol
PD	panic disorder
PET	positron emission tomography
SPECT	single photon emission tomography
SSDNA	salmon sperm DNA
SSRIs	selective serotonin reuptake inhibitors
TBE	tris-borate-EDTA buffer
TBPS	t-butylbicyclophosphorothionate
TE	tris-EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
TLC	silica gel thin layer chromatography

1. General Introduction[*]

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[*] A version of this chapter has been submitted for publication: Tanay V.A.-M.I., Bradwejn J., Baker G.B. and Bateson A.N. (1998) Possible pathophysiological imbalance in panic disorder: Focus on GABA and CCK.

Generalized anxiety disorder, obsessive-compulsive disorder, post-traumatic stress disorder, social phobia and panic disorder (PD) are examples of pathological human anxiety. PD is characterized by recurrent panic attacks which are discrete episodes of intense fear associated with cognitive and somatic symptoms (DSM-IV, 1994). The somatic symptoms are suggestive of an activation of the neurovegetative system associated with panic attacks, and include dyspnea, palpitations, dizziness, nausea, choking and paresthesia, whereas the cognitive symptoms frequently experienced include the fear of dying, fainting, losing one's mind or losing control. The classification of PD as a separate anxiety disorder is relatively recent: the distinction between PD and generalized anxiety was made in DSM-IIIR and confirmed in DSM-IV. However, this disorder is not a recent psychiatric illness. The history of PD can be traced back over more than 100 years. Indeed, descriptions of acute anxiety attack were made during the French Revolution and recently, evidence has been brought forward that Charles Darwin may have suffered from PD (Barloon and Noyes, 1997; Angst, 1995).

1.1. EPIDEMIOLOGY OF PANIC DISORDER

PD is relatively common, with a lifetime prevalence ranging from 1.2 to 3% in the general population (Weissman, 1990; Weissman, 1993; Katschnig and Amering, 1990; Angst, 1995; Katerndahl and Realini, 1993). The incidence is not equal between the two genders: female patients are almost twice as numerous as males (Weissman, 1990; Katschnig and Amering, 1990; Wittchen and Essau, 1993; Katerndahl and Realini, 1993; Roth, 1996). The overall mean age of onset is 25 years, but the female mean age of onset is later by 2-4 years than that of males (Woodman, 1993; Dick et al., 1994; Roth, 1996). Although many epidemiological studies were conducted in the United States, the data for Edmonton fall in the average range with a lifetime prevalence rate of 1.2%, and again females are twice as likely as males to develop PD, but 2 years later on average (Bland et al., 1988; Dick et al., 1994).

PD is often associated with self-perception of poor health, marital problems, increased financial dependency, risk of alcohol abuse, increased medication and suicide attempts (Dyck et al., 1988; Markowitz et al., 1989; Weissman, 1990; Wittchen and Essau, 1993; Leon et al., 1995; Roth, 1996; Angst, 1995; Massion et al., 1993; Cox et al., 1994; Appleby, 1994; Warshaw et al., 1995). Furthermore, PD is frequently related to a long record of stress, anxiety and difficulties in relationships (Angst, 1995; Roth, 1996;

Wittchen and Essau, 1993), it follows a chronic course and it rapidly evolves into a complex network of avoidance behaviors (Wittchen and Essau, 1993; Roth, 1996; Bradwejn, 1993a). The economic cost of this disorder, due to an increase in the use of health care services and lost workdays, can be dramatically reduced by proper diagnosis and efficacious psychiatric treatment (Leon et al., 1995; Markowitz et al., 1989; Salvador-Carulla et al., 1994; Salvador-Carulla et al., 1995).

Familial and twin studies (Weissman et al., 1993; Weissman, 1993; Maier et al., 1993; Woodman, 1993), as well as the Epidemiologic and Catchment Area survey (Markowitz et al., 1989; Leckman et al., 1990; Weissman, 1990), have implicated a genetic transmission of the disorder and highlighted the comorbidity of PD with other psychiatric disorders such as major depressive disorder, substance abuse or agoraphobia (Wittchen and Essau, 1993; Dick et al., 1994; Reich et al., 1993). Co-occurrence of PD with depression is associated with an earlier onset with more severe symptoms, poorer treatment outcome and increased psychosocial impairment (Weissman, 1990; Weissman et al., 1993; Reich et al., 1993; Grunhaus et al., 1994; Lydiard, 1995; Andrade et al., 1996). Although several candidate genes were investigated, genetic studies excluded a simple mode of inheritance by failing to support a linkage between a single genetic marker and the inheritance of PD (Woodman, 1993; Anonymous, 1996; Schmidt et al., 1993).

The pharmacotherapy of PD is based on a long-term treatment model to maximize clinical improvement and involves the use of a variety of drugs with differing apparent primary mechanisms of action such as tricyclic antidepressants, monoamine oxidase inhibitors, selective serotonin reuptake inhibitors (SSRIs) and benzodiazepines (Ballenger, 1986; Ballenger, 1991; Schweizer et al., 1993; Black et al., 1993; Burrows et al., 1993; Salzman, 1993; Pecknold, 1993; Curtis et al., 1993; Rosenberg, 1993; Layton and Dager, 1994; Westenberg, 1995; Westenberg, 1996; Milrod and Busch, 1996). In addition, multiple high daily dosages may be required to alleviate the recurrent anticipatory anxiety and the rebound anxiety that emerges between benzodiazepine doses (Herman et al., 1987; Bradwejn, 1993a; Westenberg, 1995).

1.2. ANIMAL MODELS OF PANIC DISORDER

As a working tool, animal models of human disorders are often sought; their validation, however, relies on the fulfillment of specific criteria. A given animal model will have a predictive validity if the drugs used in the clinical situation are effective in the model; it will have a face validity if it mimics the neuroanatomical, biochemical, physiological and pharmacological aspects of the disorder; finally it will have construct validity if homologous constructs can be studied both in the model and in the clinical situation (Commissaris and Fontana, 1991). Regarding PD, an animal model will display good predictive validity if: (1) the array of pharmacologically effective agents reflects the variety of the clinical PD treatments; (2) similarly to the clinical situation, chronic treatment with antipanic agents is required before any effect occur; and (3) established panicogenic agents administered acutely display opposite effects to the antipanic agents. So far, the strategy for anxiety and panic animal models has been to study the effects of pharmacological agents (antipanic or panicogenic) on anxiety- or fear-like behaviors of laboratory animals (predictive and face validity) rather than to try to model specific changes in affect associated with panic attacks (construct validity). Such pharmacological validation of an animal model of anxiety relies on (1) re-establishing a behavior that is suppressed by aversive stimulus, or (2) attenuating a behavior that is enhanced by aversive conditions, or (3) reversing a pharmacologically induced anxious state. The summary of the different animal models and the effects of drugs in these behavioral paradigms is presented in the Table 1.1. below.

1.2.1. Potential predictive validated models

1.2.1.1. Reversal of suppressed behaviors

The restoration of a suppressed behavior often implies that the animal is conditioned, e.g. by means of punishment applied beforehand. The conditioned suppression of drinking paradigm has been proposed as a model for PD, since the conditioned inhibition of the drinking behavior was reversed by chronic treatment with the non-selective monoamine oxidase inhibitor, phenelzine, the tricyclic antidepressants, imipramine, desipramine and amitryptiline, as well as the benzodiazepine, alprazolam (Commissaris and Fontana, 1991). In addition, other antidepressants (bupropion, mianserin) or anxiolytics (phenobarbital, chlordiazepoxide, propranolol) do not display any anxiolytic-like effects at doses which have been shown in other behavioral tests to produce antidepressant or anxiolytic effects (Commissaris and Fontana, 1991). Surprisingly, clonidine and the panic-inducing agent, yohimbine, display some anxiolytic-like properties in the conditioned suppression of drinking paradigm (Commissaris and Fontana, 1991).

Alternative non-shock conflict paradigms such as the elevated plus-maze test, the social interaction task, or the novelty-suppressed feeding, which do not require the conditioning of animals, do not present the same predictive validity (Commissaris and

Fontana, 1991; Miczek et al., 1995). Although yohimbine displays anxiogenic properties in both the elevated plus-maze and the social interaction paradigms, the two antipanic agents phenelzine and imipramine were reported to be ineffective in both paradigms (Commissaris and Fontana, 1991). However, it was recently demonstrated that phenelzine displays anxiolytic effects in the elevated plus maze at doses at which it elevates brain GABA levels (Paslawski et al., 1996). In addition, panic-inducing challenges with sodium lactate infusion and exposure to carbon dioxide do not produce any anxiogenic-like effects in the elevated plus-maze (Commissaris and Fontana, 1991). In this test, the $GABA_A$ receptor agonist, muscimol, the benzodiazepine receptor agonist diazepam and valproic acid (GABA-transaminase inhibitor) display anxiolytic properties (Dalvi and Rodgers, 1996). In contrast, the $GABA_A$ receptor antagonists, bicuculline and picrotoxin, enhance anxious behavior, and the $GABA_B$ receptor ligands (baclofen) and the GABA reuptake inhibitor, No-711, show little or no effect (Dalvi and Rodgers, 1996). In novelty-suppressed feeding, chronic treatment with desipramine or amitriptyline produced anxiolytic-like effects (Commissaris and Fontana, 1991). However, these models rely on the assessment of the exploratory behavior, which is not a simple measure of anxiety but is rather dependent on the combination of neophobia and the exploratory drive which itself is influenced by multiple external and internal factors (Harro et al., 1995).

1.2.1.2. Reversal of potentiated behaviors

Aversive manipulations performed on conditioned or unconditioned animals can potentiate other behaviors such as the startle response to acoustic stimuli or defensive burying (Miczek et al., 1995). Yohimbine displays anxiogenic-like properties in both paradigms (Commissaris and Fontana, 1991). However, the potentiated startle response is also selectively affected by anxiolytics such as barbiturates, benzodiazepines, and buspirone-like anxiolytics, but not by chronic imipramine treatment (Commissaris and Fontana, 1991). Data support the pharmacological validation of the shock-probe burying test for the study of fear and anxiety, but not for panic-like behaviors (Treit et al., 1994). Indeed, acute treatment with barbiturates or benzodiazepines dose-dependently reduces the burying behavior (Treit et al., 1994; Commissaris and Fontana, 1991), but chronic treatment with imipramine, desipramine or pargyline does not produce any anxiolytic-like effects (Commissaris and Fontana, 1991).

1.2.1.3. Reversal of pharmacologically-induced behavior

Some agents such as pentylenetetrazol or β-carboline carboxylic acid ethyl ester (β-CCE) can induce anxious behaviors, such as agitated responses and distress vocalizations which are reduced by most anxiolytics (Miczek et al., 1995). These pharmacologically-validated models are therefore good predictors for the treatment of generalized anxiety, but surprisingly they show poor abilities to identify drugs for other types of anxiety disorders such as PD (Heninger, 1989; Zacharko et al., 1995).

1.2.2. Potential face validated models

Other approaches have aimed at the establishment of paradigms modeling aspects of the changes observed in PD. For example, $GABA_A$ receptor blockade by bicuculline methiodide microinjection in the cardiostimulatory region of the dorsomedial hypothalamus elicited somatic (cardiovascular and respiratory) as well as behavioral responses associated with intense anxiety states similar to human panic attacks (Shekhar, 1994). The cardiovascular, respiratory and anxiogenic effects elicited by bicuculline methiodide microinjection were inhibited by chronic treatment with two antipanic agents with different primary mechanisms of action: imipramine and clonazepam (Shekhar, 1994). The similarities between these somatic and behavioral responses elicited in rats and the human symptoms of panic attacks allow extrapolation of these findings to human PD. In a subsequent study using the same somatic and behavioral parameters, impairment of dorsomedial hypothalamic GABA transmission by chronic infusion of a glutamic acid decarboxylase (GAD) inhibitor enhanced the physiological arousal induced by sodium lactate infusion (Shekhar et al., 1996). These data further support the involvement of GABA and the dorsomedial hypothalamus in panic-like behaviors in rats, since the results parallel the clinical situation where PD patients display enhanced physiological responses to lactate infusion compared to healthy controls (Papp et al., 1993). Finally, the somatic and behavioral responses elicited by the stimulation of the dorsal periaqueductal gray (PAG) also resembles some aspects of panic attacks (Jenck et al., 1995). In this model, drugs precipitating (caffeine, yohimbine, or cholecystokinin - CCK-) or reducing (clonazepam, alprazolam, or imipramine) panic attacks were found to dose-dependently enhance or decrease, respectively, the aversive effects of dorsal PAG stimulation (Jenck et al., 1995; Mongeau and Marsden, 1997a; Mongeau and Marsden, 1997b).

1.2.3. Potential construct validated models

A major challenge to experimental research on anxiolytics is the quantification of the psychological dimension of anxiety. One approach is to model the affective expressions (such as fear) under socially relevant conditions as a way to provide an insight into the neurobiology of anxiety. The socially relevant conditions include social separation, social conflicts, or aggressive confrontations (Miczek et al., 1995). The conditioned ultrasonic distress vocalization of rats exposed to arousing situations is reduced by a variety of anxiolytic agents (Molewijk et al., 1995). The fact that anxiolytic drugs devoid of antipanic activity such as buspirone also produce a behavioral effect argue against the screening specificity of this test for PD (Molewijk et al., 1995). An alternative paradigm is the mouse defense test battery which assesses defensive reactions in these animals when exposed to a natural predator (in this case a rat). Chronic administration of two antipanic agents, imipramine and fluoxetine, decreased the flight responses and defensive attack behaviors (Griebel et al., 1995).

1.2.4. Summary of animal models

The predictive validity of the anxiety paradigms studying the reversal of a suppressed behavior is highly variable. Whereas the plus-maze and the social interaction tests, highly responsive to anxiolytics, show no sensitivity to the effects of chronic antipanic treatment, the conditioned suppression of drinking and the novelty-suppressed feeding are both responsive to chronic administration of antipanic drugs. However, further studies with panicogenic agents are still needed to complete the validation of these models. The predictive validity of models based on the reduction of enhanced behavior (shock-probe burying test, startle response) seems also limited in PD, since chronic antipanic treatment does not produce any effects. The approaches of Shekhar (Shekhar, 1994) and Jenck (Jenck et al., 1995) are steps toward a face validated model of PD and may provide a basis for understanding the etiology of PD. However, these studies need to undergo a more complete pharmacological validation. Finally, the study of anxious behavior in socially relevant situations offers an interesting alternative approach; however these paradigms do not display any strong validity for PD since they are responsive to many anxiolytics. These animal models help emphasize that although panic attacks are characterized by somatic and behavioral responses which can be assessed, important diagnostic criteria that differentiate PD from generalized anxiety involve the subjective appreciation and reporting of cognitive symptoms which cannot be recorded from the animals. Consequently, it can be argued that the parameters assessed from these animal models may be more relevant to anxiety than PD. Finally, PD is also typified by the recurrence of spontaneous panic attacks occurring without triggering cues; these spontaneous fear-like behaviors so far are absent from the proposed animal models.

Model validity	**Behavioral model**	**Ineffective treatments**	**Effective treatments**
Predictive	Reversal of suppressed behaviors		
	Conditioned suppression of drinking	bupropion mianserin phenobarbital chlordiazepoxide propranolol	phenelzine imipramine amitryptiline alprazolam clonidine yohimbine
	Elevated plus maze	phenelzine? imipramine sodium lactate CO_2 baclofen No-711	yohimbine phenelzine? muscimol diazepam valproic acid bicuculline picrotoxin
	Social interaction	phenelzine imipramine	yohimbine
	Novelty-suppressed feeding		desipramine amitryptiline
	Reversal of potentiated behaviors		
	Startle response	imipramine	yohimbine barbiturates benzodiazepines buspirone
	Defensive burying	imipramine desipramine pargyline	yohimbine barbiturates benzodiazepines

	Reversal of pharmacologically-induced behaviors		
	Pentylenetetrazol or β-carboline infusion		most anxiolytics
Face	GABAergic blockade in the dorsomedial hypothalamus		imipramine clonazepam sodium lactate
	Dorsal periaqueductal gray stimulation		caffeine yohimbine clonazepam alprazolam
Construct	Ultrasonic distress vocalizations		most anxiolytics (including buspirone)
	Mouse defense battery test		imipramine fluoxetine

Table 1.1 Summary of proposed animal models for panic disorder and their response to the drugs tested.

1.3. NEUROBIOLOGY OF PANIC DISORDER

The cardiovascular and respiratory symptoms indicative of the involvement of the autonomic nervous system in panic attacks and the animal models of PD have suggested the involvement of several neuroanatomical locations in PD. Clinical and pre-clinical neurobiological studies have further investigated the possible role of several brain nuclei with regard to their possible link to the main neuropathophysiological theories of PD.

1.3.1. Pathophysiological models

The two main theories of the etiology of PD implicate an autonomic hyperactivity involving the locus coeruleus (LC) or an abnormality of the respiratory centers. In the early years of PD research, an increased central noradrenergic activity was repeatedly associated with the induction of panic. Evidence for an abnormal regulation of the noradrenergic system which may result in PD has been reviewed extensively (Charney et al., 1990a). The noradrenergic neurons of the LC have been the focus of attention since the brainstem location of this nucleus makes it a candidate for triggering the cardiovascular and respiratory responses observed in panic attacks. However, as discussed below and reviewed by Johnson et al. (1995) and Zacharko et al. (1995), data linking PD to a noradrenergic system abnormality are controversial. Further complexity arises from the fact that the LC function is regulated by numerous neuronal systems such as the endogenous opiates, serotonin, acetylcholine, GABA, adrenaline, corticotropin releasing factor and substance P (Charney et al., 1990b).

The data linking PD to carbon dioxide hypersensitivity and hyperventilation have also been reviewed (Papp et al., 1993). According to this respiratory theory, PD patients would have a lower suffocation firing threshold of their respiratory centers, and hyperventilation would be an adaptive mechanism to maintain CO_2 levels below threshold and prevent panic attacks (Papp et al., 1993; Klein, 1995). Although PD patients and their first-degree healthy relatives are more sensitive to CO_2 inhalation and lactate infusion than healthy controls (Gorman et al., 1987; Gorman et al., 1994; Perna et al., 1995b), not all patients suffering from this disorder experience panic attacks upon pharmacological challenge. This would suggest that PD may represent a heterogeneous group of disorder subtypes. However, failure to reveal differences in the familial transmission of PD between lactate sensitive and insensitive patients (Reschke et al., 1995) excludes the possibility of lactate sensitivity as a screening test for a subtype of this disorder.

1.3.2. Anatomical considerations

In order to pinpoint the brain activation foci involved in panic attacks and anxiety, cerebral blood flow recordings by Positron Emission Tomography (PET) studies were performed during infusions with panicogenic agents such as lactate (Reiman et al., 1989) or CCK (Benkelfat et al., 1995). Significant increases in cerebral blood flow in the temporal lobes, the insular-claustrum-lateral putamen region, as well as the cerebellar vermis were reported (Reiman et al., 1989; Benkelfat et al., 1995). The increases in cerebral blood flow in the temporal lobes were suggested to be due to increased temporal artery blood flow since superimposition of the PET with MRI images revealed that the increase in blood flow was peripheral to the temporal lobes (Benkelfat et al., 1995). However, these authors did not speculate on the observed increases in cerebral blood flow observed in the insular-claustrum region or the cerebellar vermis (Benkelfat et al., 1995). Consistent with the presence of a PD pathophysiological process located at the brainstem level, the amplitudes of the waves III and V of brainstem auditory evoked potentials under basal conditions are larger in PD patients than in healthy volunteers

(Knott et al., 1994), and the interpeak intervals of waves III and V are altered upon lactate challenge (Knott and Lapierre, 1986). Another study reported an increased occurrence of vestibular dysfunction in PD patients with agoraphobia (Jacob et al., 1996). In contrast to the clinical studies, pre-clinical experiments implicate a number of brain structures, such as the amygdala, pons-medulla, thalamus, hippocampus, LC, and PAG as mediators of the broad range of behaviors and physiological responses associated with anxiety and fear (Graeff, 1994; Zacharko et al., 1995).

Animal defensive behaviors are composed of several graded levels (the end point of which is the fight-or-flight response) that correspond to the activation of different brain structures. A body of evidence suggests that the fight-or-flight behavioral response, which is related to the rage/panic emotion, may be controlled by the PAG (Graeff, 1994). Among the vertically organized brain structures involved in the regulation of defensive behavior, the amygdala, which functions as a sensory-emotional integrator (Morris et al., 1996), transfers information to the hypothalamus and the PAG which in turn select and organize the appropriate behavioral and physiological reactions. The integrator function of the amygdala may be the result of the cytochemical organization of this nucleus. For example, the central extended amygdala contains GABAergic neurons which receive cortical inputs, and that synapse onto neurons projecting to the nucleus tractus solitarius (NTS) and the dorsal vagal motor nucleus (Sun et al., 1994). Thus, GABAergic neurons of the central amygdala are at the interface between cortical afferents and brainstem projection neurons (Sun et al., 1994). At the level of the amygdala, the axon terminals originating from the NTS and ventrolateral medulla also receive an extensive GABAergic innervation (Jia et al., 1997). Further, the central nucleus of the amygdala projections to the NTS and the ventrolateral medulla are predominantly GABAergic (Jia et al., 1997). These projections to the intermediate NTS, that receives cardiorespiratory afferents at the level of the area postrema, target GABA-immunoreactive and non-immunoreactive dendrites, but many of these dendrites received convergent input from axons that were intensely labeled for GABA (Pickel et al., 1996). Lesions of the amygdala and/or the hypothalamus do not affect the response generated by the PAG, and the cardiovascular pattern of defense elicited from the hypothalamus remains after the lesion of the PAG matter (Graeff, 1994). This suggests that these different structures are involved in parallel circuits influencing the cardiovascular functions.

Evidence suggests that the dorsomedial hypothalamus is intimately involved in the central regulation of respiration and may be associated with changes resulting in abnormal sensitivity of the medullary respiratory centers (Shekhar, 1994). Further, the dorsomedial hypothalamus has a close interaction with the raphé nuclei, the NTS, the PAG and the LC. Thus, the dorsomedial hypothalamus and the PAG are potential sites of panic production, and knowledge of the physiological roles of these areas may increase our understanding of both the pathophysiology of panic attacks and animal defensive behavior. Consistent with the pathophysiological theories of PD, many of these preclinical data suggest abnormalities at the brainstem level. In order to reconcile the proposed pathophysiological theories of PD with the neuroanatomical data, neurotransmitter interconnections and neuroregulation of the nuclei associated with PD and fear behaviors will be examined.

Brainstem LC neurons project primarily to the cortex, tectum, cerebellum and the dorsal horn of the spinal cord (Grzanna and Fritschy, 1991; Jones, 1991). The LC also innervates select sensory and association nuclei of the brainstem such as the brainstem reticular formation, the sensory trigeminal complex, the principal nucleus of the inferior olive, the cochlear nuclei, the NTS, the pontine nuclei, the interpeduncular nucleus and the tectum of the midbrain (Grzanna and Fritschy, 1991; Jones, 1991). Although noradrenergic neurons receive limited innervation onto their soma and proximal dendrites, their long distal dendrites extend beyond the limits of the nucleus, allowing them to receive projections from multiple regions (Jones, 1991; Nistico and Nappi, 1993). Hence, LC neuron dendrites receive afferents from the paragigantocellularis lateralis and the prepositus hypoglossi of the medulla and from fibers traveling from the forebrain down to the PAG and dorsolateral pontine tegmentum (Nistico and Nappi, 1993; Jones, 1991). The perisomatic GABAergic innervation of the LC is of moderate density and derives in part from the periventricular gray, the tegmentum, the vestibular nucleus, the prepositus hypoglossi nucleus, as well as from the few interneurons present within the LC (Jones, 1991; Nistico and Nappi, 1993; Shefner and Osmanovic, 1991). LC neurons possess both $GABA_A$ and $GABA_B$ receptors, the activation of which decreases the activity of LC neurons (Shefner and Osmanovic, 1991; Nistico and Nappi, 1993).

Evidence reviewed by Bradwejn (1993b) and Gorman et al. (1989) suggests that the projections from the NTS to the nucleus paragigantocellularis and subsequently to the LC may be involved in the control of sympathetic nerve discharge which in turn regulates the cardiovascular and respiratory systems. Reciprocally, the NTS receives afferent fibers from many neurovegetative centers such as the cardiovascular and respiratory centers (Palkovits and Zaborszky, 1977). The majority of the visceral afferents from the cardiovascular, respiratory and gastrointestinal systems terminate in the caudal two-thirds of the nucleus (Maley, 1996). Taking advantage of the high affinity of [^{3}H]-aspartate for the excitatory amino acid reuptake system, the retrograde transport of this radiolabel microinjected in the NTS identified a convergent network of pathways originating form various brain regions (Beart et al., 1994). Excitatory afferents to the NTS stem from the paraventricular hypothalamic, the PAG, the medial parabrachial, the trigeminal, the vestibular, the reticular, the inferior olive and all raphé nuclei (Beart et al., 1994). In addition, another study has identified glutamatergic projections from the insular cortex to the rostrocaudal NTS (Torrealba and Muller, 1996). In contrast, inhibitory afferents have been identified by [^{3}H]-GABA retrograde labeling, originating from discrete brain areas such as the trigeminal nucleus, the dorsal part of the medullary reticular formation, the parvocellular reticular nucleus, the facial nucleus, the nucleus ambiguus and the raphé obscurus (Beart et al., 1994). Within many of the subnuclear regions of the NTS, GABA immunoreactive puncta are evenly distributed in the neuropil (Maley and Newton, 1985) which preferentially surrounds the proximal regions (cell somata, proximal or intermediate dendrites) of both GABA immunoreactive and non-immunoreactive neurons (Torrealba and Muller, 1996; Maley, 1996). Therefore, GABA immunoreactivity can be attributed to both GABA interneurons present within the NTS and GABA neurons projecting onto the NTS (Maley, 1996).

In the PAG, the combination of retrograde labeling with immunohistochemical localization of GABA-containing neurons revealed that the GABAergic innervation of PAG-raphé and raphé-PAG projection neurons is abundant (Williams and Beitz, 1990). Significant GABAergic-GABAergic connections are also observed in the PAG, and GABA$_A$ receptor immunoreactivity is present along the membrane and perikarya of GABAergic and non-GABAergic neurons (Williams and Beitz, 1990). Evidence suggests that some of the benzodiazepine anxiolytic effects are mediated by their action at GABA$_A$ receptors located in the PAG (Schenberg and Graeff, 1978; Russo et al., 1993). Finally, the brainstem inferior colliculus has been associated with the brain aversive system which is thought to include the amygdala, the medial hypothalamus and the dorsal PAG. Administration or application of benzodiazepine or GABA$_A$ receptor agonists to the inferior colliculus reduced the escape behavior triggered by electrical stimulation of this acoustic structure (Melo et al., 1992), suggesting that the antiaversive action of these drugs may be mediated via GABA$_A$ receptors.

These neurobiological models of PD both suggest possible abnormalities at the brainstem level, but the available data do not attribute the pathophysiology of PD to a unique alteration in a specific brain structure. On the contrary, the investigations of the physiological mechanisms of fear and anxiety states provide strong support for the involvement of multiple brain neurochemical systems, including noradrenergic, GABAergic, serotonergic, and corticotropin-releasing hormone receptors, in the pathophysiology of human anxiety. The variety of pharmacotherapies available and the possible neuroanatomical origins of PD have led to the development of different neurotransmitter hypotheses of this disorder. The shortcomings of the first two neurotransmitter models, involving noradrenaline and serotonin, have resulted in the emergence of GABAergic and CCK models of PD. The current trend is now to approach the pathophysiological process of PD in an integrative way, taking into account the close interactions between different neurotransmitter systems (Zacharko et al., 1995). Evidence for the main neurotransmitter theories of PD will be considered here, in terms of their possible interactions with one another.

1.4. THE MONOAMINE HYPOTHESES OF PANIC DISORDER

Biological evidence for noradrenergic, serotonergic and dopaminergic dysfunctions in PD have recently been reviewed in considerable detail by a number of authors (Johnson et al., 1995; Zacharko et al., 1995). The first study suggesting that the LC was involved in the expression of fear in monkeys (Redmond and Huang, 1979) led to many studies investigating the role of noradrenergic or serotonergic transmission in anxiety and PD (Rosenberg, 1993; Layton and Dager, 1994; Zacharko et al., 1995; Sheehan et al., 1993; Miczek et al., 1995; Heninger, 1989). The noradrenergic hypothesis of PD stemmed from studies suggesting that an abnormal α2-adrenoceptor system is specific to patients suffering from this disorder (Charney et al., 1990b; Charney et al., 1992). Indeed, PD patients displayed a greater sensitivity to the α2-adrenergic antagonist, yohimbine, in panic attack rates as well as in increases in heart rate, blood pressure and in plasma levels of the noradrenaline metabolite, 3-methoxy-4-hydroxyphenylglycol (MHPG) compared to healthy control subjects (Charney et al., 1990b; Charney et al., 1992). Conversely, PD patients were more sensitive to the decreasing effect of clonidine on plasma MHPG (Charney et al., 1992; Coplan et al., 1997), but presented a blunted rise in growth hormone level following clonidine administration (Charney et al., 1992). These data suggested a diminished postsynaptic α2-adrenoceptor function concomitant with a presynaptic noradrenergic neuronal hyperactivity. One study reported a decrease in heart rate in PD patients after administration of the β-adrenoceptor agonist, isoproterenol (Charney et al., 1990a), whereas other studies have found a decrease in β-adrenoceptor density on blood mononuclear cells and lymphocytes and a lower isoproterenol-stimulated cAMP production in lymphocytes of PD patients (Heninger, 1989; Maddock et al., 1993). Thus, it has been postulated that PD may be linked to a decrease in postsynaptic β-adrenoceptor density and function resulting from chronic activation mediated by a presynaptic noradrenergic activity (Charney et al., 1990a). Finally, higher baseline plasma MHPG levels and heart rate have been suggested as predictors of poorer antipanic treatment outcome amongst PD patients (Slaap et al., 1996), and another study linked clinical response to greater decreases in basal MHPG levels and a decreased plasma MHPG variation upon clonidine challenge (Coplan et al., 1995; Coplan et al., 1997). Hence, noradrenergic dysfunction may characterize a subgroup of PD patients in whom positive treatment outcome may relate to a normalization of the noradrenergic system.

Evidence has been accumulating to support the involvement of serotonin in human and animal anxiety (Heninger, 1989; Pecknold, 1990) but, as reviewed by Pecknold (1990), some findings have not been consistent among research groups. The interactions between serotonergic and noradrenergic systems may be relevant to the manifestation of anxiety in that the activity of the serotonergic synapses was shown to be an important determinant for the magnitude of the growth hormone response to clonidine (Pecknold, 1990) and thus for the activity of the noradrenergic neurons. Further, the raphé serotonergic nuclei receive projections from, and send projections to, the LC. Data reporting a reduced serotonin-induced platelet aggregation and serotonin uptake into platelets as well as an elevated 5-HT$_2$ receptor density in PD patients (Butler et al., 1992), support the postulated decreased serotonergic transmission in anxiety and PD. The view is that a decreased synaptic serotonin concentration is responsible for a reduction in the inhibitory effects of serotonin on the firing of the LC neurons. Accordingly, SSRIs alleviate CCK-tetrapeptide- (CCK$_4$) and CO$_2$-induced panic attacks (Van Megen et al., 1997a; Pols et al., 1996), and the antipanic efficacy of SSRIs is similar to the tricyclic antidepressants, but the reduced side effect profile of the SSRIs renders them better tolerated (Lecrubier and Judge, 1997; Lecrubier et al., 1997). However, long-term administration of 5-HT$_{1A}$ receptor agonists showed a beneficial effect in an open trial with PD patients (Pecknold et al., 1993) and no effect in double-blind placebo-controlled studies (Sheehan et al., 1990; Sheehan et al., 1993). Chronic treatment with these agents is supposed to lead to a desensitization of the presynaptic inhibitory 5-HT$_{1A}$ receptors located on raphé serotonergic neurons and therefore an enhanced firing of the raphé neurons and synaptic serotonin levels (Artigas et al., 1996). The fact that long-term administration of 5-HT$_{1A}$ receptor agonists did not consistently produce beneficial effects in PD patients does not support the theory that PD pharmacotherapy is primarily linked to an elevation of synaptic serotonin concentrations.

Although the serotonergic hypothesis may provide an explanation for the efficacy of antidepressants that increase serotonin levels in PD, chronic treatment with these agents is usually characterized by a biphasic response consisting of an initial worsening of the symptoms before any clinical improvement is reported (Ballenger, 1991; Layton and Dager, 1994). This initial exacerbation of symptoms after acute administration, in a time frame which corresponds to the rapid effect of antidepressants on monoaminergic neurotransmission, is in contrast to what one would expect if abnormalities in the monoaminergic systems were primarily involved in the pathophysiology of PD. The delayed onset of therapeutic action of these antidepressant/antipanic drugs (Jefferson, 1997) leads to the speculation that the onset of antipanic therapy may be a consequence of a remodeling of neurotransmission.

1.5. GABAERGIC SYSTEM AND PANIC DISORDER

GABA is the major inhibitory neurotransmitter in the vertebrate central nervous system (CNS). Widely distributed throughout the CNS, GABA modulates the action of several neurotransmitters, such as noradrenaline and serotonin, which have been implicated in various mood disorders, and PD in particular (Scatton et al., 1986; Petty, 1995; Zacharko et al., 1995; Suranyi-Cadotte et al., 1990; López-Rubalcava et al., 1992). Therefore, GABA is good candidate for being a mediator of the dysregulations triggering PD and/or of the therapeutic effects of antipanic agents. Further, GABA exerts a tonic inhibitory regulation on the PAG and LC, brainstem nuclei suspected to play an important role in PD and associated defensive behaviors (Charney et al., 1990a; Graeff, 1994). Indeed, GABA tonically regulates the dorsal PAG so that the antiaversive response in animals elicited by electrical stimulation of the PAG is enhanced by injections of GABAergic antagonists ($GABA_A$ receptor antagonists or GABA synthesis inhibitors) or attenuated by GABAergic transmission facilitators (benzodiazepines, barbiturates) (Graeff et al., 1993; Graeff, 1994). In addition, Priolo et al. (1991) reported that microinjections of $GABA_A$ receptor antagonists into the LC evoked somatic and electrocortical panic-like responses. Further, $GABA_A$ receptor-mediated inhibition of some brain structures such as the LC is stronger when the postsynaptic cell is depolarized (Shefner and Osmanovic, 1991), suggesting that this phenomenon may constitute a feedback mechanism for the GABAergic control of cell excitability. Consequently, an abnormality of GABA transmission resulting from alterations in this neurotransmitter's level and/or or receptor number/function, and leading to a decreased control of cell excitability may underlie PD etiology.

1.5.1. Neurotransmitter levels

In contrast to healthy women that exhibit an increase in plasma GABA levels from the mid-follicular to the late menstrual phase, a decrease in plasma GABA levels over the same time period was reported in women suffering from premenstrual dysphoric disorder (Halbreich et al., 1996). However, decreased plasma GABA concentrations appear to be a biological marker for mood disorders such as bipolar illness or major depression but not for PD, bulimia or schizophrenia (Petty et al., 1990; Petty et al., 1993a; Goddard et al., 1996). In accordance with these data, Roy-Byrne et al. (1992) examined GABA plasma levels before and after chronic treatment with the antipanic drug, alprazolam, and did not observe any pre-treatment differences between the panic patients and their controls, or within the panic patients group between pre- and post-treatment levels. Similarly no change in plasma GABA levels was observed in patients with major depression treated for 4 weeks with desipramine (a major metabolite of imipramine and an antidepressant in its own right) (Petty et al., 1993b). These various negative results could have been explained by the lack of correlation observed between plasma and cerebrospinal fluid (CSF) GABA in different species (rat, cat, dog and human) and the suggestion that the latter may be more appropriate for monitoring brain GABA (Löscher, 1979). Rimón et al. (1995) did not observe any significant difference between CSF GABA levels in panic patients and healthy controls before treatment, and after chronically treating these patients with either alprazolam or imipramine. However these researchers reported that low pretreatment levels of CSF GABA correlated significantly with poor therapeutic response to these two drugs.

Two commonly used antipanic drugs, imipramine and phenelzine, both increase GABAergic transmission. Imipramine has been reported to increase GABA release from the thalamus (Korf and Venema, 1983), whereas phenelzine causes a marked, long-lasting elevation of hypothalamus and brain GABA (Baker et al., 1991; Paslawski et al., 1995) which appears to be due, at least in part, to inhibition of GABA transaminase (GABA-T) (Popov and Matthies, 1969; McManus et al., 1992; McKenna et al., 1994). Although imipramine increases GABA release *in vitro*, 4 week treatment with desipramine (a metabolite of imipramine which is also an antidepressant) did not alter cortex GABA concentrations in rats (McManus et al., 1992). These long-term studies should be put into the perspective of the time frame necessary to obtain clinical improvement in PD. Indeed, 2 to 4 weeks of drug administration are generally required before any therapeutic effect is recorded in PD. Therefore, the discrepancies between the central effects of imipramine on GABA release and the lack of effect on peripheral and central GABA levels suggests the involvement of other primary mechanisms, such as altered receptor number and/or function as mediator of the antipanic effects of imipramine, as well as the possible involvement of compensatory mechanisms such as those involving the GABA-transporter and metabolism enzymes.

Diazepam-binding inhibitor (DBI) is a peptide consisting of 104 amino acids which is able to displace $GABA_A$ receptor benzodiazepine site ligands with an inverse agonist activity (Costa and Guidotti, 1991; Pelissolo, 1995). CSF DBI concentrations did not differ in PD patients and controls (Payeur et al., 1992), but a significant correlation between CSF levels of DBI and corticotropin-releasing factor levels was found (Costa and Guidotti, 1991; Payeur et al., 1992). Since a single determination of CSF DBI concentrations was performed between panic episodes (Payeur et al., 1992), abnormal DBI secretion related to panic attacks may not have been detected. DBI secretion may, like corticotropin-releasing hormone release, be episodic and correlate with panic attacks. Similarly to a benzodiazepine-site negative modulator, episodic DBI release may, like the β-carbolines (Calogero et al., 1988), increase corticotropin release which in turn activates the hypothalamo-pituitary-adrenal axis and induces anxiety.

The data available to date does not strongly support an hypothalamo-pituitary-adrenal axis dysregulation in PD. In an early study, PD patients presented a blunted adrenocorticotropic hormone (ACTH) response to corticotropin-releasing hormone challenge (Roy-Byrne et al., 1986), whereas more recent data indicated that PD patients do not show blunted ACTH or cortisol responses to corticotropin-releasing hormone challenge (Curtis et al., 1997). In addition, CSF corticotropin-releasing hormone levels are not different in PD patients compared to controls (Jolkkonen et al., 1993), and chronic treatment with alprazolam, which decreases corticotropin-releasing hormone concentrations in the LC of rats (Owens et al., 1993), does not alter CSF corticotropin-releasing factor levels in PD patients (Jolkkonen et al., 1993). Similarly, CSF corticotropin-releasing factor levels remain unchanged in PD patients after chronic imipramine administration (Jolkkonen et al., 1993), whereas evidence suggests that desipramine blocks the central noradrenergic control of the hypothalamo-pituitary-adrenal axis (Torpy et al., 1995). In accordance with these last two studies, data suggest that an uncoupling of the noradrenergic-hypothalamo-pituitary-adrenal axis, characterized by a lack of correlation between MHPG and cortisol levels, is a marker of the disorder and not altered by antipanic treatment (Coplan et al., 1995).

1.5.2. GABA receptors

1.5.2.1. Molecular biology of GABA receptors

1.5.2.1.1. $GABA_A$ receptors

Radioligand binding studies have shown that $GABA_A$ receptors are widely distributed throughout the mammalian CNS (Enna and Snyder, 1975). $GABA_A$ receptors are members of a ligand-gated ion channel superfamily and are thought to be composed of five subunits arranged around a central pore forming the ion channel (Nayeem et al., 1994). The receptor heterogeneity arises from the presence of different subunits which are encoded by separate genes. These subunits display a high degree of amino acid sequence identity and are suggested to share a common genetic ancestor (Ortells and Lunt, 1995). A schematic of the $GABA_A$ receptors is presented in Figure 1.1.

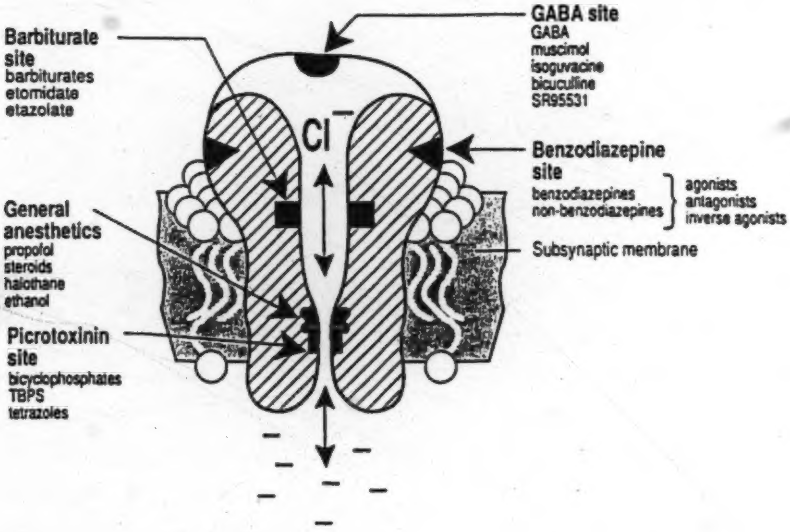
Barbiturate site
barbiturates
etomidate
etazolate
GABA site
GABA
muscimol
isoguvacine
bicuculline
SR95531
Cl⁻
Benzodiazepine site
benzodiazepines
non-benzodiazepines
agonists
antagonists
inverse agonists
General anesthetics
propofol
steroids
halothane
ethanol
Subsynaptic membrane
Picrotoxinin site
bicyclophosphates
TBPS
tetrazoles

Figure 1.1 Schematic of the $GABA_A$ receptor
This figure taken from Richards et al. (1991) depicts the chloride ion channel associated with the $GABA_A$ receptor, and arbitrarily presents the different allosteric recognition sites that modulate the receptor function.

To date, 6α-, 3β-, 3γ-, 1δ-, 3ρ and 1ε-subunits have been identified in the mammalian brain, and further subunit heterogeneity arising from the alternative splicing of primary subunit transcripts generates a potentially huge heterogeneity among GABA$_A$ receptors (Sieghart, 1995; Zhang et al., 1995; Wilke et al., 1997; Ogurusu and Shingai, 1996). So far, the precise subunit composition of a receptor *in vivo* is not known, but mRNA co-localization (Laurie et al., 1992; Wisden et al., 1992) and immunoprecipitation (Quirk et al., 1994b; Quirk et al., 1994a; McKernan and Whiting, 1996; Macdonald and Olsen, 1994; Sieghart, 1995; Jechlinger et al., 1998) and immunohistochemistry (Fritschy and Mohler, 1995; Fritschy et al., 1998; Somogyi et al., 1996; Nusser et al., 1998; Nusser et al., 1996) studies have suggested several possible GABA$_A$ receptor subunit combinations in particular brain regions (reviewed by McKernan and Whiting, 1996; Dunn et al., 1994): e.g., α1β1γ2 in visual cortex (Huntsman et al., 1994), α2β1/3 and α5β1/3 in hippocampus (Wisden et al., 1992; Persohn et al., 1992), α1α3β2/3γ2 in cortex (Persohn et al., 1992; Araujo et al., 1996), α6α1β2/3γ2/δ in cerebellar granule cells (Laurie et al., 1992; Wisden et al., 1992; Persohn et al., 1992).

Over the years, a number of agents have been found to influence GABA$_A$ receptor function, rendering the pharmacology of the GABA$_A$ receptor complex (Sieghart, 1995). GABA-induced effects mediated by GABA$_A$ receptor activation are mimicked by muscimol and competitively inhibited by bicuculline, which both act at the GABA binding site (Sieghart, 1995). In addition to the binding site for GABA, GABA$_A$ receptors also display modulatory sites for the benzodiazepines, the anticonvulsant barbiturates, some neurosteroids, and the channel blockers, picrotoxin and t-butylbicyclophosphorothionate (TBPS) (Sieghart, 1995). Every site is able to interact with one or more of the other sites, so that binding at one site can affect the binding properties of another (Tallman et al., 1978; Sigel and Barnard, 1984). Barbiturates increase the mean channel open time (Study and Barker, 1981), and steroids increase the affinity of GABA positive modulators for the GABA site (Harrison et al., 1987) and the mean channel open time (Simmonds and Turner, 1987). Benzodiazepines increase the frequency of channel opening (Study and Barker, 1981) but not the burst duration (Macdonald and Twyman, 1992) as well as increase the affinity of GABA agonists for the GABA low affinity binding site (Skerritt et al., 1982b; Skerritt et al., 1982a). Finally, other agents such as alcohol are also able to modulate GABA$_A$ receptor function; although, except at lethal concentrations, radioligand binding studies have not established a direct interaction of ethanol with any of the above-mentioned sites (Ticku, 1990).

Transient expression systems have been used to show that different subunit combinations confer different functional properties on the resulting receptor subtypes (Pritchett et al., 1989a; Pritchett et al., 1989b; Dunn et al., 1994; Sieghart, 1995). The contribution of the different subunit isoforms to the receptor properties is summarized in Table 1.2. The α subunit plays a major role in the determination of channel function properties and the recognition characteristics of the GABA site (Ebert et al., 1994). The β subunit which contributes to the GABA binding site (Bureau and Olsen, 1985) has little influence on the GABA recognition site, but is important in determining the characteristics of the channel response to GABA (Sigel et al., 1990; Ymer et al., 1989). The presence of a γ-subunit affects GABA gating of the channel (Sigel et al., 1990). The replacement of the γ2 by the γ3 subunit markedly enhances the affinity of agonists for the GABA site (Ebert et al., 1994), whereas the exchange of the γ2 for the γ1 subunit alters both the channel current maximal amplitude and the EC_{50} of GABA agonists at the recombinant receptors (Ebert et al., 1994; Ducic et al., 1995). However, the direction of the change is influenced by both the α and β subunit isoforms present. Finally, electrophysiological data provide evidence for a physiological and functional role for the different receptor subtypes (Pearce, 1993). In hippocampal slice preparations, CA_1 neurons' response decay to $GABA_A$-receptor mediated inhibitory postsynaptic potentials displays bi-exponential kinetics (Pearce, 1993). The fast (3-8 ms) and slow (30-70 ms) current decay components are anatomically segregated (fast component near or at the cell body, slow component away from the cell body) and present different shifts in reversal potential, supporting the idea that the activation of different receptor subtypes may mediate these responses (Pearce, 1993).

The benzodiazepine site of $GABA_A$ receptors is recognized by 3 types of modulators: positive (agonists), negative (inverse agonists) or blockers (antagonists) (Sieghart, 1995). In a similar manner, the benzodiazepine pharmacology of $GABA_A$ receptors is also affected by the subunit composition of the recombinant receptors (Table 1.2.). Hence, the α subunit plays a critical role in defining the binding characteristics of the benzodiazepine site (Pritchett et al., 1989a; Pritchett and Seeburg, 1990). The functional and binding characteristics of the recombinant receptor expressing different α-subunit isoforms rationalized the pharmacological classification of the recombinant receptors. Type I receptors display high affinity for the triazolopyridazine Cl 218,872 and some β-carboline-3-carboxylate esters and are simulated by the receptors expressing the α1βxγ2 subunit combination (Pritchett et al., 1989a). Type II receptors have a lower affinity for Cl 218,872 and are associated with receptors expressing α2βxγ2, α3βxγ2 or α5βxγ2 subunit combinations (Pritchett et al., 1989a). A third type of receptor, corresponding to the receptors expressing α4β2γ2 or α6β2γ2 subunit combinations, is called diazepam-insensitive since diazepam and other classical agonist benzodiazepines do not bind to this receptor subtype, whereas the benzodiazepine Ro 15-4513 binds to these receptors with high affinity (Lüddens et al., 1990). The presence of a γ subunit is essential for generating GABA $_A$ receptor responses to the application of benzodiazepines (Sigel et al., 1990). The replacement of the γ2 by the γ3 subunit markedly decreases the affinity of the receptor for the benzodiazepine site agonist (Herb et al., 1992), whereas the exchange of the γ2 for the γ1 subunit reduces the affinity of all benzodiazepine site ligands, especially that of flumazenil and DMCM (methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate) (Ymer et al., 1990). The replacement of the γ2 by the γ3 subunit also reverses the negative modulation of Ro 15-4513 and dimethoxy-4-ethyl-β-carboline-3-carboxylate to a positive effect (Puia et al., 1991). In contrast, the β-subunit isoforms have little influence on the benzodiazepine recognition site but play a crucial role in the coupling between the benzodiazepine and TBPS binding sites (Sigel et al., 1990; Verdoorn et al., 1990).

Subunit subtype	**GABA $_A$ receptor properties affected**
α	Channel function GABA binding site Benzodiazepine binding site
β	Channel response to GABA Benzodiazepine-channel sites coupling
γ	Channel response to GABA GABA binding site Benzodiazepine binding site

Table 1.2 Overview of the GABA $_A$ receptor properties affected by different subunit subtypes

GABA $_A$ receptor subunit gene expression is modulated during development and aging (Zheng et al., 1993; Baumgartner et al., 1994; Mathews et al., 1994; Hu and Ticku, 1994) by synaptic activity (Huntsman et al., 1995; Huntsman et al., 1994) and by NMDA or GABA $_A$ receptor stimulation (Poulter et al., 1997; Harris et al., 1995). In addition to the rapid desensitization and the sequestration of the receptor, GABA $_A$ receptor plasticity also arises from a receptor up- or down-regulation which is accompanied by changes in the receptor properties and subunit gene expression (Miller et al., 1988). GABA $_A$ receptor subunit gene expression is modulated physiologically and pharmacologically by exposure to GABA $_A$ receptor ligands. As illustrated below, the differential alteration in the expression of the subunits is drug- and subunit-specific, as well as brain region- and time-dependent. Hence chronic administration of diazepam decreases, but chronic treatment with FG7142 increases, α1-subunit mRNA levels in the cortex (Heninger et al., 1990; Primus and Gallager, 1992), while [^{3}H]-Ro 15-1788 benzodiazepine binding in cortex is decreased by chronic exposure to flurazepam and increased by chronic administration of FG7142 (Pritchard et al., 1991; Miller et al., 1988). In addition, chronic treatment with ethanol decreases α1- and increases α6-subunit mRNA and polypeptide levels in the cerebellum (Wu et al., 1995). Chronic exposure to FG 7142 elevates α1-subunit mRNA levels and benzodiazepine binding site density in the hippocampus but not in the cerebellum (Primus and Gallager, 1992; Pritchard et al., 1991). Finally, aging Fisher rats show a decrease in TBPS binding site density and in α1-subunit mRNA levels in the cortex (Mhatre and Ticku, 1992a).

1.5.2.1.2. GABA$_B$ receptors

This second type of GABA receptor is also widely distributed in the CNS, but the cerebellum is one of the brain regions containing the highest concentration of GABA$_B$ receptors (Kerr and Ong, 1995). GABA$_B$ receptors belong to the G-protein coupled receptor superfamily (Kerr and Ong, 1995; Kaupmann et al., 1997). The activation of GABA$_B$ receptors has been linked to the inhibition of adenylyl cyclase activity and phosphatidylinositol turnover, as well as the modulation of ion channels (Nakayasu et al., 1992; Nakayasu et al., 1993; Ohmori and Kuriyama, 1989; Kuriyama et al., 1993; Dunlap and Fischbach, 1981; Dunlap and Fischbach, 1978; Newberry and Nicoll, 1985; Newberry and Nicoll, 1984; Kerr and Ong, 1995). Activation of GABA$_B$ receptors mediates a reduction of transmitter release and causes hyperpolarization of neurons. Presynaptically, the inhibition of neurotransmitter release is achieved by a decrease in Ca^{2+} channel conductance (Dunlap and Fischbach, 1981; Dunlap and Fischbach, 1978), while an increase in K$^+$ channel conductance mediates a long-lasting postsynaptic hyperpolarization (Newberry and Nicoll, 1984; Newberry and Nicoll, 1985; Kerr and Ong, 1995). Finally, many neurochemical and functional studies have reported a phaclofen-sensitive inhibition of GABA release by GABA$_B$ autoreceptor-mediated mechanisms in various brain areas such as the spinal cord, cortex or hippocampus (Anderson and Mitchell, 1985; Bonanno and Raiteri, 1993a; Bonanno and Raiteri, 1993b; Hughes et al., 1993; Kerr and Ong, 1995). Recently two GABA$_B$ receptor cDNAs were cloned (Kaupmann et al., 1997). The corresponding proteins present the characteristics of G-protein coupled receptors, and differ from one another by a sequence stretch located in the N-terminal domain, suggesting that these may be isoforms of an alternatively spliced primary transcript (Kaupmann et al., 1997). Expression studies revealed that both proteins are negatively coupled to adenylyl cyclase and give rise to receptors displaying similar pharmacological profiles (Kaupmann et al., 1997).

1.5.2.2. GABA receptors and PD

Both types of GABA receptor have been examined for their potential role in PD pathophysiology and/or pharmacotherapy. Only one open clinical trial has tested and reported the possible treatment of PD subjects with the selective $GABA_B$ receptor agonist baclofen (Breslow et al., 1989). The potential involvement of $GABA_B$ receptors in PD treatment is further questioned by the fact that chronic treatment of rats with various antipanic drugs including imipramine, phenelzine and desipramine does not alter cortical $GABA_B$ receptor binding (Engelbrecht et al., 1994; McManus and Greenshaw, 1991). However, the potential antipanic activity of $GABA_B$ receptor agonists may be complex in that pharmacological and functional evidence supports the notion of heterogeneous tissue distribution and synaptic location of $GABA_B$ receptors (Gemignani et al., 1994; Kerr and Ong, 1995).

In contrast, several pieces of evidence indicate that $GABA_A$ receptor may be the target for antipanic drugs. In correlation with the respiratory pathophysiological theory of PD, it was recently shown that oxygen and CO_2 levels alter $GABA_A$ receptor binding properties. Indeed, hyperbaric hyperoxia decreased the number of cortical central benzodiazepine receptors (Courtiere et al., 1991), whereas CO_2 inhalation increases [^{35}S]-TBPS binding in the cortex, cerebellum and hippocampus (Concas et al., 1993). Further, pretreatment with alprazolam prevented the CO_2 inhalation-induced increase of [^{35}S]-TBPS binding (Concas et al., 1993). Neurosteroids are endogenous modulators of $GABA_A$ receptors (Majewska, 1992; Sieghart, 1995). In order to elucidate the biological basis for the higher prevalence of PD in women, the sensitivity of female PD patients to CO_2 inhalation was investigated during their menstrual cycle. The anxiogenic effects of CO_2 were increased in the early-follicular phase compared to the midluteal phase (Perna et al., 1995a). Interestingly, recent studies have shown that the amplitude of potentiation of GABA-gated Cl- influx depend both on the neurosteroid present and the brain region investigated (Wilson and Biscardi, 1997), and that, although $GABA_B$ receptors are mainly involved in the regulation of GABA release, in restricted brain areas $GABA_A$ receptor-induced GABA release is dependent on the presence of gonadal hormones (Fleischmann et al., 1995). Therefore, variation in the hormonal balance during the menstrual cycle may alter $GABA_A$ receptor-mediated neuronal inhibition and GABA release over time. Consequently, a reduction in GABA release and neuronal inhibition in specific brain nuclei may contribute to the precipitation of panic attacks during certain menstrual phases, such as the early-follicular phase which is characterized by low levels of sex hormones.

Chronic treatment with desipramine, clorgyline or paroxetine decreased cortical $GABA_A$, but not $GABA_B$, receptor binding site density following bulbectomy (Dennis et al., 1994). Chronic desipramine treatment reduced the number of binding sites for [^{3}H]-flunitrazepam in the forebrain and the $GABA_A$ receptor open channel blocker [^{35}S]-TBPS in the hippocampus of rats (Suranyi-Cadotte et al., 1990), and decreased the effect of flurazepam application on CCK-induced activation of hippocampal pyramidal neurons (Bouthillier and deMontigny, 1987). However, some controversy arises from the reports (Kimber et al., 1987; Todd et al., 1995) that chronic administration of phenelzine or imipramine (as well as desipramine, trancylpromine or zimeldine) fails to alter the density or affinity of benzodiazepine binding sites in the rat cortex, as measured by [^{3}H]-flunitrazepam binding. In light of the known heterogeneity of $GABA_A$ receptors, such a negative finding can be explained by the fact that flunitrazepam, like other classical benzodiazepines, recognizes $GABA_A$ receptors containing $\alpha 1$, $\alpha 2$, $\alpha 3$ or $\alpha 5$ and $\gamma 2$ subunits. Therefore any substitution between these α subunits within the existing receptors, or any alterations in the number of $\alpha 4$- or $\alpha 6$-subunit containing receptors may not be detected by flunitrazepam. In contrast, TBPS, that binds to all $GABA_A$ receptor subtypes, will not reveal any subunit substitution within the receptors present, but will unveil any alteration in the total number of receptors. Hence, if the total number of receptors containing $\alpha 4$-, $\alpha 6$- or no γ-subunit varies, [^{3}H]-flunitrazepam binding will remain unchanged whereas [^{35}S]-TBPS binding will be altered.

The benzodiazepines are known to produce their CNS effects by interacting with specific allosteric recognition sites located on $GABA_A$ receptors (White et al., 1981; Sethy and Harris, 1982; Sethy et al., 1983; Martin, 1987; Richards et al., 1991; Sieghart, 1995; Dunn et al., 1994). Amongst the anxiolytic benzodiazepines used to treat PD, alprazolam and clonazepam are commonly prescribed (Liebowitz et al., 1986; Ballenger, 1986; Hollister, 1986; Ballenger et al., 1988; Schweizer et al., 1993; Rosenberg, 1993; Bradwejn, 1993a; Burrows et al., 1993; Layton and Dager, 1994), but more recently diazepam has been proposed as an alternative to alprazolam treatment (Noyes et al., 1996). The antipanic dosage of benzodiazepines (for example alprazolam) is markedly higher than the recommended dosage for generalized anxiety disorder (Dunner et al., 1986; Alexander and Alexander, 1986; Sheehan et al., 1993; Bradwejn, 1993a; Rosenberg, 1993; Salzman, 1993), but in contrast to some of the other therapeutic properties of benzodiazepines (Miller et al., 1988; Miller et al., 1989), tolerance does not seem to develop to the antipanic effects of these drugs (Pollack et al., 1986; DuPont et al., 1992; Curtis et al., 1993; Lepola et al., 1993; Bradwejn, 1993a; Rosenberg, 1993; Layton and Dager, 1994). PD patients do, however, develop tolerance to the side effects of benzodiazepines (such as sedation, amnesia, fatigue and motor impairment), and physical dependence and withdrawal syndromes are commonly reported (Ballenger, 1991; Bradwejn, 1993a; Rosenberg, 1993; Salzman, 1993; Pecknold, 1993; Burrows et al., 1993; Layton and Dager, 1994).

Although only a few benzodiazepines have been recognized as effective antipanic agents, reports that PD may be associated with a decreased GABA$_A$ receptor sensitivity (Roy-Byrne et al., 1989; Roy-Byrne et al., 1990; Cowley et al., 1995; Malizia et al., 1995; Roy-Byrne et al., 1996) led investigators to study the characteristics of peripheral benzodiazepine receptors and GABA$_A$ receptors in panic patients. The peripheral benzodiazepine receptor is found in both central and peripheral tissue (Anholt et al., 1985). This single subunit receptor (Sprengel et al., 1989; Krueger et al., 1990) is located in the outer mitochondrial membrane (Anholt et al., 1986) which is recognized by DBI (Costa and Guidotti, 1991). The peripheral benzodiazepine receptor is thought to be involved in the regulation of cholesterol transport, the rate-limiting step of steroidogenesis (Papadopoulos et al., 1990; Mukhin et al., 1989; Costa and Guidotti, 1991), and to play a role in the regulation of the transport of oxidative metabolism substrates (Moreno-Sanchez et al., 1991). Hence the peripheral benzodiazepine receptor seems to be part of a regulatory step for the synthesis of endogenous steroids, some of which, in turn, modulate GABA$_A$ receptor function (Costa and Guidotti, 1991). Evidence suggests that the number of binding sites for this receptor is reduced in platelets of PD patients compared to that of healthy controls (Marazziti et al., 1994).

Single photon emission tomography (SPECT) studies using an iodinated benzodiazepine receptor antagonist ligand (iomazenil) showed abnormal benzodiazepine receptor binding in PD patients (Schlegel et al., 1994; Kuikka et al., 1995; Kaschka et al., 1995). However, these studies did not generate consistent results in terms of specific regional increases or decreases in benzodiazepine receptor binding. The fact that flumazenil is anxiogenic and induces panic attacks in patients with PD but not in controls, suggests that benzodiazepine binding site function may be altered towards the inverse agonist direction in PD (Nutt et al., 1990). According to this view, classical benzodiazepine receptor antagonists such as flumazenil behave as partial inverse agonists in PD patients (Malizia et al., 1995) and therefore display a pharmacological shift from blocking to negative modulation of $GABA_A$ receptor function in these patients. However, in a recent clinical study flumazenil did not induce panic attacks in PD patients whom were sensitive to sodium lactate (Ströhle et al., 1998). Although these data do support the idea that abnormal $GABA_A$ receptor function underlie PD, these authors could not rule out that the non-response to flumazenil of their lactate-sensitive group may characterize a special subgroup (Ströhle et al., 1998). It is known that not all PD patients are sensitive to lactate (Gorman et al., 1987; Keck et al., 1993; George et al., 1995; Cowley et al., 1996), therefore it is possible that flumazenil-sensitive PD patients include some that are non-responsive to lactate. Given that the efficacy and affinity of GABA and benzodiazepines for the $GABA_A$ receptor depend on the receptor subunit composition, an alteration in $GABA_A$ receptor subunit composition could be the cornerstone of the etiology of PD in some PD patients. Under such circumstances, GABA and $GABA_A$ receptor agonists would have reduced efficacy, leading to a decrease in the inhibitory neurotransmission mediated by GABA, which in turn could create an imbalance between a normal excitatory and a weakened inhibitory neurotransmission.

Finally, valproate has been demonstrated to possess some antipanic efficacy in several open trials (Primeau et al., 1990; Woodman and Noyes, 1994; Keck et al., 1993) and in a double-blind placebo controlled study (Lum et al., 1990). In addition, a case report of the efficacy of the combination of sodium valproate and clonazepam in refractory PD patients who were resistant to antidepressant treatment (Ontiveros and Fontaine, 1992) is consistent with this hypothesis. Although the action of sodium valproate is not completely understood, it has a GABA-enhancing action (Macdonald and Bergey, 1979). An indirect effect of valproate on GABAergic transmission may be by means of increasing glutaminase and decreasing glutamine synthetase activities (Collins et al., 1994), which could in turn enhance the transformation of glutamate into GABA. Indeed, sodium valproate increases GABA levels in CSF (Löscher, 1979) and in selective brain regions (Löscher and Hörstermann, 1994), but has no effect on brain glutamate or plasma GABA levels (Löscher, 1979; Löscher and Hörstermann, 1994). Therefore the combination of clonazepam with sodium valproate apparently potentiates GABAergic transmission via two different mechanisms: clonazepam by acting at the benzodiazepine site of $GABA_A$ receptors, and sodium valproate by increasing brain GABA levels and consequently the amount of GABA available for synaptic transmission. Such a combination would be advantageous if PD is linked to an alteration in $GABA_A$ receptor subunit composition, because a synergistic action between clonazepam and sodium valproate may restore GABA transmission inhibitory tone. Hence, it would be of interest to determine if such a combination could have some therapeutic advantages for PD patients such as an earlier onset of action or an effectiveness in refractory cases.

1.6. OTHER NEUROBIOLOGICAL HYPOTHESES

1.6.1. Cholecystokinin and panic disorder

CCK is a peptide found in high concentrations in the cerebral cortex, basal ganglia, nucleus accumbens, olfactory tubercle, PAG and limbic system of the mammalian CNS (Woodruff et al., 1991; Raiteri et al., 1993; Rehfeld and Nielsen, 1995). CCK is present at intermediate to high levels in many brain regions (cortex, hippocampus, amygdala, striatum, hypothalamus, lateral septal nuclei, PAG and area postrema) with the exception of the cerebellum (Rehfeld and Nielsen, 1995). The cholecystokinin gene consists of 3 exons (Deschenes et al., 1985) which encode a prepropeptide of 115 aminoacids further modified and cleaved to generate several bioactive peptides (for a review see Rehfeld and Nielsen, 1995). Sulfated CCK$_8$ peptide, which contains the 8 C-terminal amino acids of the CCK polypeptide, constitutes the majority of the active CCK peptides in the cerebral cortex, but several larger peptides containing the sulfated and C-terminus CCK$_8$ sequence have also been extracted from the cortex (Rehfeld and Nielsen, 1995). Finally, smaller fragments of CCK$_8$ (CCK$_4$ and CCK$_5$) of similar affinity for CCK$_B$ receptors as CCK$_8$ have also been identified in the cortex (Rehfeld and Nielsen, 1995). Two types of receptors, denoted CCK$_A$ and CCK$_B$, have been identified. CCK$_A$ receptors are found in the gastrointestinal tract and in discrete brain regions, while CCK$_B$ receptors are found throughout the CNS (Mercer and Beart, 1997; Mercer et al., 1996; Hill et al., 1990). In addition to their differential distribution, these receptors are characterized by separate pharmacologies (Woodruff and Hughes, 1991)

The possible involvement of CCK in anxiety disorders originated from microiontophoresis studies by Bradwejn and de Montigny (1984). These authors found that various benzodiazepines administered intravenously or applied by microiontophoresis antagonize CCK$_{8S}$-induced excitation of hippocampal pyramidal neurons of rats and flumazenil, a classical benzodiazepine receptor antagonist, attenuates the antagonism caused by benzodiazepines. Further studies by Bradwejn and de Montigny (1985) suggested that the antagonism by benzodiazepines of CCK-induced activation of the hippocampal neurons by rats is related to their anxiolytic properties.

Injection of CCK_8 at very low doses into the amygdala of rats has been reported to enhance arousal and fear-related motivation behaviors (Fekete et al., 1984). Intravenous administration of CCK_4 to African green monkeys elicited behaviors consistent with fear/panic-like responses, including vigilance, agitation, restlessness, and, at peak levels of intensity of the reactions, immobility or freezing (Ervin et al., 1991). Pretreatment with alprazolam or a specific CCK_B receptor antagonist attenuated the effects of the CCK_4 challenge (Ervin et al., 1991). Rehfeld (1992) reported that symptoms of anxiety, choking and unreality (symptoms suggestive of panic attacks) were produced by CCK_4 in healthy human volunteers. A series of clinical studies have since shown that administration of CCK_4 to humans produces panicogenic effects, that PD patients are more sensitive than normal controls to these effects and that these patients equate the induced panic attacks to their usual ones (Bradwejn, 1993b; Bradwejn et al., 1994a). Finally, Bradwejn and coworkers (Bradwejn, 1993b; Bradwejn and Koszycki, 1994) recently reported that the antipanic drugs imipramine and alprazolam antagonize the panicogenic effects of CCK_4 in PD patients.

1.6.1.1. CCK in animal models of PD

Many studies reported little or no anxiolytic properties for CCK receptor antagonists in anxiety paradigms in which conflict is created by punishment (Dooley and Klamt, 1993; Dawson et al., 1995; Charrier et al., 1995; Harro et al., 1995; Rodgers and Johnson, 1995; Van Megen et al., 1996a). However, a more recent investigation showed that the CCK_B receptor antagonist, LY288513, reduced the conditioned freezing induced by inescapable electric footshock (Izumi et al., 1996). In contrast, in alternative non-shock conflict paradigms, CCK receptor agonists decreased the exploratory behavior (locomotor activity and time spent in the open compartment) in the elevated plus-maze or in the exploration box tests (Rex et al., 1994; Harro et al., 1995; Rodgers and Johnson, 1995; Van Megen et al., 1996a; Matto et al., 1997a; Biro et al., 1997; Bickerdike et al., 1995; Rex et al., 1997). These anxiogenic effects were prevented by pre-treating the animals with the selective CCK_B receptor antagonist L-365,260 (Rex et al., 1994; Rodgers and Johnson, 1995; Matto et al., 1997b; Matto et al., 1997a). Consistent with the involvement of CCK in anxiety, CCK peptides increase defensive probe burying in rats (Harro et al., 1995; Rodgers and Johnson, 1995; Van Megen et al., 1994; Van Megen et al., 1996a) or the acoustic startle response (Fendt et al., 1995; Josselyn et al., 1995). Finally, the CCK_B receptor antagonist L-365,260 reduces the startle response potentiated by fear (Josselyn et al., 1995).

In construct validated models, such as the dorsal PAG stimulation, the CCK_B receptor antagonist L-365,260, but not CI-988, another CCK_B receptor antagonist, displays some antipanic-like properties (Jenck et al., 1996). The lack of behavioral response after CI-988 or CCK_4 (CCK_B receptor agonist) administration was suggested to reflect the poor brain penetration of these compounds (Jenck et al., 1996). However, this study may parallel the clinical situation where the antipanic dosage is orders of magnitude higher than the anxiolytic dose, and hence the effective dose may not have been reached in this study. Finally, in the social separation paradigm assessing ultrasonic distress vocalization, CCK_4 increases the number of distress calls of young rat pups separated from their mother (Harro et al., 1995). The implication of the central nucleus of the amygdala in the brain aversive system, does not seem to involve CCK neurotransmission since intra-amygdala injection of CCK fragments did not affect the number of entries into or the time spent on the open arms of the elevated plus maze (Huston et al., 1998).

Altogether, the effects of CCK_B receptor ligands in the shock-probe burying test are consistent with an involvement of CCK in anxiety. The effects of CCK_B receptor ligands are more pronounced in the plus maze and social interaction tests than in the conditioned suppression of drinking and novelty suppressed feeding paradigms. Because of the extensive pharmacological screening performed, the predictive validity of the conditioned suppression of drinking for antipanic properties is high. The negative and discrepant results with the CCK_B receptor ligands may be due to the poor brain permeability of some of them (Jenck et al., 1996). Finally, the discrepant findings regarding the efficacy of CCK_B receptor ligands and antipanic drugs reported for some paradigms may be due to the fact that the paradigms used do not assess anxiety directly, but rather the variation in some behavioral consequences (i.e. behavioral suppression, exploratory behavior) which depends extensively on the anxiety baseline of the animal and may reflect alterations in the levels of specific aspects of anxiety.

1.6.1.2. Peptide levels in PD

To investigate possible CCK abnormalities in CNS, the levels of the peptide were determined in PD patients and normal subjects. CSF CCK_8 concentrations were significantly lower in patients with PD than in normal subjects (Lydiard, 1994). Brambilla et al. (1993) measured lymphocyte CCK_8 concentration in PD patients and controls before and after a 30 day course of alprazolam therapy. The CCK_8 concentrations were significantly lower in PD patients than in the controls and did not change after alprazolam therapy (Brambilla et al., 1993). A possible common effect of antipanic drugs on CCK transmission is suggested by the fact that treatment with the antipanic drugs clomipramine and clonazepam for 14 days increased CCK-like immunoreactivity levels in the ventral tegmental area and the cingulate cortex (Brodin et al., 1994). However a 3 week treatment with another antipanic agent, imipramine, did not alter the levels of the different molecular forms of CCK in rat frontal cortex (Harro et al., 1997). It is therefore possible that the effect of antipanic drugs on CCK transmission is a regional phenomenon.

1.6.1.3. CCK receptors

1.6.1.3.1. Molecular biology of CCK receptors

CCK acts at two types of receptors that possess distinct pharmacologies: CCK_A and CCK_B receptors. CCK_A and CCK_B receptors share 48% sequence identity and are predicted to possess 7 transmembrane domains characteristic of G-protein coupled receptors (Wank et al., 1992). Indeed, in neostriatal neurons, the excitatory effects of CCK_B receptor agonists are mediated by a pertussis-insensitive G-protein and the phosphatidyl inositol second messenger pathway which leads to the enhancement of membrane cationic conductance (Wu and Wang, 1996). Recently, evidence has been presented supporting the existence of at least two CCK_B receptor isoforms generated by alternative splicing of a common primary transcript (Jagerschmidt et al., 1994). The majority of CCK receptors in the CNS are of the CCK_B type. The CCK_A receptors are found in discrete brain nuclei such as the area postrema, nucleus solitarius and interpeduncular nucleus (Bradwejn, 1993b; Woodruff and Hughes, 1991; Mercer et al., 1996; Hill et al., 1990). However, recent data suggest that the distribution of CCK_A receptors may be more widely spread than originally described (Mercer and Beart, 1997). cDNAs for these receptors have recently been cloned (Wank et al., 1992).

1.6.1.3.2. CCK receptors and PD

The anxiogenic effects of CCK challenges may result from a direct or indirect action at CCK receptors located in brainstem structures such as the NTS, the area postrema or dorsal raphé (Bradwejn, 1993b; Woodruff and Hughes, 1991). These brainstem regions are interconnected by diverse neuronal projections and are linked to adrenergic structures (such as the LC) thought to play a major role in PD (Bradwejn, 1993b). Postsynaptic CCK_B receptors of NTS neurons are excitatory, whereas postsynaptic CCK_A receptors are inhibitory (Branchereau et al., 1992a). Functional heterogeneity of CCK_B receptors has been reported. Indeed, CCK_B receptor agonists differentially modulate dopamine release from the anterior part of the nucleus accumbens (Léna et al., 1997).

The relative involvement of CCK_A and CCK_B receptors in PD has also been explored. In a double-blind, placebo-controlled study, administration of the CCK_B receptor antagonist L-365,260, prior to challenging PD patients with CCK_4, significantly reduced the number and intensity of symptoms, as well as the frequency of panic attacks induced by CCK_4 (Bradwejn et al., 1994b). Further, since CCK_A receptor antagonists do not produce anxiolytic effects except at high doses, the involvement of CCK_B receptors (rather than CCK_A receptors) is suspected in anxiety (Woodruff et al., 1991). The fact that pentagastrin, another CCK_B receptor ligand, can also induce panic attacks in panic patients similar to those induced by CCK_4, not only provides another model of panic, but further argues for the role of CCK_B receptor systems in human anxiety (Abelson and Nesse, 1994; Van Megen et al., 1994). Finally, the cardiovascular and anxiogenic effects of pentagastrin in healthy volunteers are antagonized by L-365,260 (Lines et al., 1995).

Surprisingly, a 3 week treatment with imipramine altered the binding characteristics of CCK_B receptors in hypothalamus (increased the affinity) but not in frontal cortex (Harro et al., 1997). This negative result may be due to the fact that CCK_B receptor binding densities are heterogeneous among cortical layers (Mercer et al., 1996). Therefore regional alterations in binding site charateristics may not be detectable in cortical homogenates. Finally, a recent study reported that CCK_4-induced intracellular Ca^{2+} mobilization in T-cells was higher in panic patients compared to controls, and that chronic treatment with alprazolam returned the CCK_4-induced intracellular Ca^{2+} mobilization to control levels (Akiyoshi et al., 1996; Akiyoshi et al., 1997). These results suggest that CCK_B receptor hypersensitivity may underlie PD. The fact that panic onset is significantly shortened upon a second CCK_4 challenge (Bradwejn et al., 1992) also supports such a view.

Clinical studies investigating the efficacy of CCK_B receptor antagonists to attenuate panic attacks have been disappointing. CI-988 failed to block lactate- or CCK_4-induced panic in normal volunteers (Cowley et al., 1996; Bradwejn et al., 1995) or PD patients (Van Megen et al., 1997b), nor did it decrease anxiety in generalized anxiety disorder patients (Adams et al., 1995). Further, the first report of a placebo-controlled clinical trial with another CCK_B antagonist, L-365,260, in PD patients also yielded negative results (Kramer et al., 1995). It has been suggested that there may be pharmacokinetic problems with CI-988 (Bradwejn et al., 1995), and it will be of interest to see the effectiveness of other CCK_B receptor antagonists. Alternatively, PD patients may require higher doses of, or more potent CCK_B receptor antagonists, to counteract naturally occurring or pharmacologically-induced panic attacks.

There does not seem to be a simple relationship between CCK and its anxiogenic effects since the activation of CCK_B receptors by application of CCK peptides on neurons can be excitatory (hippocampus; (Bradwejn and de Montigny, 1984)) or inhibitory (substantia nigra; (Zhang and Freeman, 1994)). However, CCK-induced panic does not seem to involve either the hypothalamic-pituitary-adrenal axis or the LC activation since the levels of plasma prolactin, cortisol and the noradrenaline metabolite, MHPG, did not differ between PD challenged with CCK_4 or saline (Van Megen et al., 1996b). CCK release from brain slices *in vitro* is enhanced by drugs which increase cAMP levels (Beinfeld, 1996), and the expression of pro-CCK mRNA in cultured cells is enhanced by noradrenaline (Harro et al., 1993), another neurotransmitter which has been implicated in PD. Finally, the pharmacological destruction of noradrenergic nerve terminal projections from the LC can cause an increase in CCK receptor binding in brain regions such as frontal cortex and hippocampus which receive noradrenergic inputs predominantly from the LC (Harro et al., 1993). No direct correlation has been established between the anxiogenic effects of CCK and the activation of the LC or hypothalamic-pituitary-adrenal axis, nor between the noradrenergic transmission tone and levels of expression of CCK or CCK receptor genes.

The mechanisms by which CCK_4 induces panic attacks are not clear and are still under study; however, complex interactions of several neurotransmitter systems (adrenergic, serotonergic, dopaminergic, peptidergic, GABA) may underlie such a process (Crawley, 1995). So far, data seem to exclude an abnormal response of the hypothalamo-pituitary-adrenal axis to CCK (Abelson et al., 1994). These observations clearly indicate a major physiological role for CCK and related peptides in the CNS and raise questions regarding the possible therapeutic role for CCK_B receptor antagonists in the treatment of PD and other anxiety disorders. As a novel approach to the treatment of anxiety, CCK_B receptor antagonists display several major advantages over the benzodiazepines. Sedation and muscle relaxation accompanying benzodiazepines treatment are absent with the currently available CCK_B receptor antagonists, even at high doses (Woodruff and Hughes, 1991). Further, CCK_B antagonists also show no anxiogenic rebound responses after withdrawal following chronic administration (Woodruff and Hughes, 1991).

1.6.2. GABA/ CCK interaction in panic disorder

Clinical challenges and biochemical data indicate that both CCK and GABA neurotransmission may play a role in the etiology or pharmacotherapy of PD. An hypothesis which would reconcile both views is that an imbalance in the interactions between these two neurotransmitters in key brain regions may be of relevance to the pathophysiology of PD. According to this view, PD could be due to abnormal cross-talk between GABA and CCK neurotransmission. Indeed, neuroanatomical and functional data support the idea that GABA/CCK interactions are of physiological significance.

1.6.2.1. Anatomical co-localization

Neuroanatomical evidence indicates that numerous brain regions implicated in fear, anxiety and the pathophysiology of PD are modulated by both GABA and CCK. Indeed, these neurotransmitters co-localize in many brain regions and neuronal cells. For example, in the cortex and hippocampus, GABA co-localizes with the vast majority of CCK-containing neurons, while only 10% of GABAergic neurons contain CCK (Gulyas et al., 1991; Van Megen et al., 1996a). Hence, although CCK can be found within other cortical nerve terminals, GABAergic synaptosomes are enriched in CCK (Hughes et al., 1993).

In situ hybridization revealed the presence of both $GABA_A$ receptor subunit and CCK mRNAs within the auditory brainstem nuclei such as the inferior colliculus (Wynne et al., 1995), while the presence of CCK_B receptors in the inferior colliculus was confirmed by autoradiography (Mercer et al., 1996). Similarly, CCK and GABA have been identified in the NTS (Maley, 1996), but the density of their respective synthesizing neurons vary according to the subnuclear region considered (Maley, 1996). CCK is present at high levels throughout the NTS, and at the level of the area postrema, the NTS contains prominent CCK immunostaining and GABA immunoreactive neurons (Maley, 1996). Concordant with the presence of CCK neuropeptide, both CCK_A and CCK_B receptors have been identified in the NTS (Hill et al., 1990; Mercer et al., 1996; Branchereau et al., 1992a). A recent study confirmed that in the NTS, CCK was acting presynaptically (but not postsynaptically) at CCK_B receptors to decrease the GABAergic transmission from interneurons (Branchereau et al., 1992b). Endogenous CCK released *in vitro* from NTS brain slices decreases the synaptic transmission from GABAergic interneurons. Therefore, the neurovegetative components of panic attacks may result from an interaction between CCK and GABA neuronal systems in the brainstem (Branchereau et al., 1992b). An interaction between GABA and CCK may also play a role in the integration of the psychological component associated with panic attacks that may occur at the level of the amygdala. Indeed, GABA and CCK largely coexist in amygdala neurons (Morales and Bloom, 1997; McDonald and Pearson, 1989). Finally, along with GABA (Williams and Beitz, 1990), moderate autoradiographic labeling of CCK_B receptors is found within the central gray (Mercer et al., 1996). In contrast, although various neuropeptides have been identified in the area of the LC, many of which modulate LC neuronal activity (Olpe and Steinmann, 1991), neither CCK effects nor the presence of CCK receptors have been reported in this nucleus (Olpe and Steinmann, 1991; Hill et al., 1990; Mercer et al., 1996).

1.6.2.2. Physiological and functional interactions

In addition to their anatomical co-localization, evidence indicates that CCK and GABAergic systems interact functionally to modulate each other's activity. Benzodiazepines reduce the excitatory effects of CCK on hippocampal neurons (Bradwejn and de Montigny, 1984; de Montigny and Debonnel, 1994), and it has been demonstrated that this action is related to their anxiolytic properties (Bradwejn, 1993b; Chopin and Briley, 1993). These effects do not wane with time, as indicated by the fact that after 14 days of administration, benzodiazepines retained their ability to attenuate the excitatory effects of CCK microiontophoretically applied on rat hippocampal neurons (Bouthillier and de Montigny, 1988; de Montigny and Debonnel, 1994). The antagonistic effect of benzodiazepines was blocked by flumazenil, suggesting the involvement of the benzodiazepine site of $GABA_A$ receptors (Bradwejn and de Montigny, 1984). The possibility, however, that the antagonistic effects of benzodiazepines and CCK may be due to a direct action of benzodiazepines on CCK receptors has been raised. Indeed, several synthetic and natural benzodiazepine derivatives are potent CCK_B receptor antagonists (Woodruff and Hughes, 1991). CCK however, does not seem to act at $GABA_A$ receptors as a negative modulator, since the antagonist flumazenil does not block the cardiovascular and behavioral effects of CCK_4 challenge in healthy volunteers (Bradwejn et al., 1994a). This suggests that the benzodiazepine site of $GABA_A$ receptors is not a mediator of the panicogenic action of CCK_4. Reciprocally, to date there have not been any reports that benzodiazepines bind to CCK receptors (Crawley, 1995).

Whereas GABA release from cortical and hippocampal synaptosomes is instantaneous and stimulus-dependent, CCK release is delayed but short-lived (Hughes et al., 1993; Raiteri et al., 1993). GABA release from GABAergic synaptosomes is negatively modulated by both $GABA_A$ and $GABA_B$ receptor ligands (Hughes et al., 1993). Cases of regulation of spontaneous neurotransmitter release by neuronal transporters have been reported (Bonanno and Raiteri, 1994). CCK is present in many GABA-synthesizing neurons in the cerebral cortex, hippocampus, basolateral amygdala and spinal cord and its release is regulated by GABA-mediated mechanisms (Abucham and Reichlin, 1991; Harro et al., 1993; Benoliel et al., 1992; Gemignani et al., 1994). However, GABA transporters are unlikely to participate in this process since these transporters do not seem to be present on CCK-releasing cortical neurons (Bonanno and Raiteri, 1994). In contrast, in human cortical synaptosomes and rat striatal slices, K^+-stimulated release of CCK-like immunoreactivity is decreased by the $GABA_B$ receptor agonists, baclofen or CGP 47656, but not by the $GABA_A$ receptor agonist, muscimol (Raiteri et al., 1993; Raiteri et al., 1996; Gemignani et al., 1994). In the spinal cord, both muscimol and baclofen inhibit CCK-like immunoreactivity release (Benoliel et al., 1992), whereas the $GABA_A$ receptor blockers, bicuculline and picrotoxin, stimulate CCK release from cortical cells in culture (Abucham and Reichlin, 1991). In addition, acute diazepam blocks stress- and yohimbine-induced (but not basal) CCK release in the frontal cortex (Nevo et al., 1996). Thus the modulation of CCK release by GABAergic-mediated mechanism can involve either one of the two GABA receptors.

Conversely, CCK_8 enhances K^+-stimulated [^{3}H]-GABA release in slices of the cortex, caudate putamen, substantia nigra and hippocampus (de Belleroche and Bandopadhyay, 1992; Perez de la Mora et al., 1993). In a similar manner, CCK_{8S} enhances both basal and electrically-evoked [^{3}H]-GABA release from striatal slices in a dose-dependent fashion (Rakovska, 1995). This effect is blocked by a CCK_B receptor antagonist and tetrodotoxin, suggesting the involvement of CCK_B receptors located on GABAergic interneurons (Rakovska, 1995). In the nucleus accumbens, CCK_{8S}-mediated increase of GABA release is blocked by the CCK_B receptor antagonist, PD 134308, but not by the CCK_A antagonist, L-364,718 (Ferraro et al., 1996). However, this CCK_A antagonist counteracts the inhibition exerted by the CCK_B receptor antagonist on CCK_{8S}-mediated GABA release (Ferraro et al., 1996). Hence, in the nucleus accumbens, the modulation of GABA release by CCK seems to involve primarily CCK_B receptors, while CCK_A receptors modulate the effects of CCK_B receptors on GABA release.

Whole cell patch clamp studies of a brain slice preparation of NTS neurons showed that the CCK_B receptor antagonist, PD 134,308, potentiated the postsynaptic inhibitory effects of exogenously applied GABA to the NTS (McLean et al., 1996). This potentiation remained in the presence of tetrodotoxin, or after reversal of the transmembrane chloride ion gradient, or after $GABA_A$ receptor desensitization (McLean et al., 1996). These results suggest that this postsynaptic potentiation may involve an interaction at the cytosolic level (modulation of chloride channel activation) or at the extracellular level (decrease of GABA turnover). Along the same lines, iontophoretic application of CCK or CCK_A and CCK_B receptor agonists onto dorsal lateral geniculate neurons induced both excitatory and inhibitory responses (Albrecht et al., 1995). In addition, some of the inhibitions induced by CCK were blocked by bicuculline, indicating that local circuit interneurons mediate part of the CCK effects on the discharge rate of geniculate neurons (Albrecht et al., 1995). Therefore CCK_B receptor agonists may be producing panic attacks by decreasing GABA inhibition.

Finally, a number of studies suggest that GABA/CCK neurotransmission interactions may occur at the gene expression level. Indeed, lesions of hippocampal GABAergic afferents changed the level of CCK mRNA in some hippocampal interneurons (Panni et al., 1994), and acute administration of the benzodiazepine site negative modulator, FG 7142, induces CCK gene expression in the amygdala and the hippocampus (Pratt and Brett, 1995). Further, acute treatment with, and 24 hr withdrawal from, diazepam increases preprocholecystokinin mRNA levels in rat hippocampus and cerebral cortex, whereas chronic diazepam administration has no effect on the levels of this transcript (Rattray et al., 1993). Finally, chronic treatment with, and 24 hr withdrawal from, diazepam increased $[^3H]$-CCK_8 binding in the primary olfactory cortex (Harro et al., 1990).

1.7. SUMMARY

There is much evidence to suggest that amongst the many possible neurotransmitter pathways, the pathology and/or pharmacotherapy of PD may involve GABAergic and/or CCK transmissions. In addition, PD may be a biologically heterogeneous condition composed of several biological subtypes in which the primary abnormality may involve a variety of different neurotransmitter systems. Since GABA and CCK transmission seem to be two inhibitory and excitatory circuits intimately inter-related and also display large interactions with many other neurotransmitters, an imbalance between these systems may be a key step in the pathogenic process leading to PD. The efficacy of antipanic drugs may involve either the correction of an abnormal pathway or the alteration of a different neurotransmitter system which compensates for the initial deficit. Thus the elucidation of the mechanisms implicated in the antipanic effects of drugs may shed light on the pathophysiology of PD and on how the various neurotransmitter systems interact with each other.

1.8. THESIS PROJECT

Clinical data indicate that panic attacks are episodes of intense fear which involve the activation of neurovegetative centers. Preclinical and clinical data suggest that GABA regulates brain centers involved in defensive or panic-like behaviors (Graeff, 1994), and that $GABA_A$ receptor function may be altered in PD patients (Nutt et al., 1990). Since GABA is the major inhibitory neurotransmitter in the mammalian CNS, one might expect that a decrease in $GABA_A$ receptor function could lead to secondary neurotransmission abnormalities due to a weakened counteraction of the effect of depolarizing agents like CCK. Evidence suggests that antipanic drugs share the common effect of increasing GABAergic transmission (Breslow et al., 1989; Baker et al., 1991; McManus et al., 1992; McKenna et al., 1994; Korf and Venema, 1983; Dennis et al., 1994; Shekhar, 1994). Further, antipanic drugs require long-term administration before any improvement occurs, suggesting that remodeling of neurotransmission via alteration in some gene expression may underlie the therapeutic action of these drugs. These observations, and the fact that ligands at $GABA_A$ receptors can alter the receptor composition and function, lead to the suggestion that modulation of the receptor properties via alteration in the subunit composition may be the pathway by which antipanic drugs produce their effects. However, the possible combinations of subunit isoforms exceed by far the actual number of channel subtypes that can be identified either pharmacologically or electrophysiologically, suggesting that the assembly of subunits into an ion channel is unlikely to be a random process (Green and Millar, 1995). Hence, there is a clear lack of appropriate pharmacological tools to identify and quantify the different $GABA_A$ receptor subtypes. Consequently, studies of $GABA_A$ receptor subtypes are rendered difficult by both the unresolved identity of the exact subunit composition and the heterogeneous expression of the receptor subunits isoforms throughout the brain. However, there is a good correlation between the receptor subunit mRNA and protein levels and their respective distribution (Mhatre and Ticku, 1994b; Fritschy and Mohler, 1995; Laurie et al., 1992; Wisden et al., 1992), suggesting that the measurement of the subunit specific mRNA levels may provide an indication of the amount of protein present, and by extrapolation, of the possible subunit combination of the $GABA_A$ receptor subtype expressed.

The evidence for the involvement of CCK in PD arises from preclinical as well as clinical studies (Woodruff et al., 1991; Bradwejn et al., 1994b; Bradwejn et al., 1990; Abelson and Nesse, 1994). PD seems to be characterized by low CCK levels and a concomitant hypersensitivity of CCK_B receptors. The fact that CCK_A and CCK_B receptors are present in brain regions involved in panic-like reactions and the regulation of cardiovascular and respiratory centers further suggests that there may be a link between CCK system abnormality and PD pathophysiology. A possible pathophysiological mechanism by which the CCK neuronal system produces the array of autonomic symptoms of panic attacks may be either by direct action at CCK_B receptors located in some key brainstem nuclei and/or by suppression of GABAergic inhibitory input. Evidence suggests that GABA and CCK system interact at the neuroanatomical and functional levels and reciprocally influence their protein expression level. Hence the postulated alteration in $GABA_A$ receptor subunit gene expression level underlying the antipanic efficacy of drugs may be concomitant with a remodeling of CCK transmission.

I have therefore studied the effects of chronic treatment with various antipanic drugs on $GABA_A$ receptor subunit mRNA levels in different rat brain regions. The drugs used were selected to represent three major classes of antipanic therapeutic agents: the monoamine oxidase inhibitor, phenelzine, the tricyclic antidepressant, imipramine, and the benzodiazepine, alprazolam. The anxiolytic, buspirone, was added to the study as a negative control for non-specific anxiolytic mediated effects. This drug is a 5-HT_{1A} receptor agonist with some affinity for dopamine D_2 receptors, but devoid of any effect at $GABA_A$ receptors (Fulton and Brogden, 1997). According to the proposed neuroanatomical model of PD, panic attacks are presumed to mirror brainstem dysfunction, while anticipatory anxiety is linked to limbic structures, and phobic avoidance to the prefrontal cortex (Gorman et al., 1989). Further, the drug efficacy of alprazolam and imipramine does not seem restricted to panic attacks but includes other important clinical components of the disorder such as anticipatory anxiety, depressive symptoms and phobic avoidance behavior (Alexander and Alexander, 1986; Charney et al., 1986; Tesar et al., 1987; Rosenberg, 1993). Thus, the study was performed in a few selected brain regions: cortex, cerebellum, and brainstem that may be the target for some of the different aspects of the antipanic therapy (Chapters 3, 4 and 5, respectively). Finally, the brainstem region was selected to conduct comparative studies between the short-term and the long-term effects of antipanic agents on $GABA_A$ receptor subunit gene expression, as well as to determine a possible long-term effect of these drugs on the CCK system (Chapter 6). The elucidation of the mechanism of action of antipanic drugs is of primary importance for the understanding of how an efficacious therapy can be achieved, as well as extending our knowledge of the neurotransmitter system interactions in the CNS.

2. Materials and Methods*

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* Portions of this chapter have been published: (Tanay et al., 1997; Bateson and Tanay, 1998; Lai et al., 1997). I am also grateful to Mrs. G. Rauw, Department of Psychiatry, for her assistance in performing the various drug delivery assays in liver and spinal cord.

2.1. MATERIALS

2.1.1. Surgical materials

1. Alzet® osmotic minipumps (2ML1, cat. no. 4710-0; 2ML2, cat. no. 4711-0; 2ML4, cat. no. 4712-0) were purchased from Alza Co. (Palo Alto, CA, USA) and used according to the manufacturer's instructions.
2. Xilocaine ointment 5% (Astra Pharma Inc., Mississauga, ON, Canada).
3. Savlon (Zeneca Pharma, Mississauga, ON, Canada; cat. no. 3010).
4. Eye and Wound Powder (Wyerth-Ayerst Canada Inc., Montréal, QUE, Canada).
5. Metophane (Janssen, Mississauga, ON, Canada).
6. Surgical blades no. 11 and handle no. 4 (Fisher Scientific, Pittsburgh, PA; cat. nos. 08-916-5B and 08-917-5 respectively).
7. Surgical clips, clip applier and clip remover (Becton-Dickinson Canada, Mississauga, ON; cat. nos. 7631, 7630, 7637 respectively).
8. Surgical blunt-end scissors (Fine Science Tools Inc., Vancouver, BC, Canada; cat. no. 14003-18).
9. Imipramine and phenelzine were purchased from Sigma (St Louis, MO, USA; cat. nos I-7379 and P-6777, respectively).
10. Buspirone (RBI, Natick, MA; cat. no. B-119).
11. Alprazolam was a generous gift from Upjohn Company (Kalamazoo, MI).
12. Diazepam was a generous gift from Hoffmann-LaRoche (Switzerland).
13. Saline: 0.9% (w/v) NaCl sterilized by autoclaving.
14. Propylene glycol.
15. Dimethylsulfoxide (-DMSO- Sigma; cat. no. D-5879).
16. All the drug solutions were sterilized through 0.45 μm nylon filters (Nalge Co., Rochester, NY; cat. no. 195-2545) prior to the filling of the osmotic minipumps.

2.1.2. RNA isolation

All chemical and biochemical reagents used thereafter were of Analar or equivalent grade, and were obtained from the following chemical product companies: BDH (Toronto, ON, Canada), Sigma (Mississauga, ON, Canada), Pharmacia Biotech (Baie d'Urfé, QUE, Canada), Gibco-BRL (Burlington, ON, Canada), Amersham (Oakville, ON, Canada), RBI (Natick, MA), Boehringer Mannheim (Laval, QUE, Canada), Fisher Scientific (Nepean, ON, Canada). Solutions were made with distilled deionized water, and RNAse free solutions were obtained by treating with diethylpyrocarbonate (DEPC 0.1% v/v). The solutions which could not be treated with DEPC were filter sterilized through 0.22 µm filters (Millipore; cat. no. SLGV025LS).

1. Diethylpyrocarbonate (-DEPC- BDH; cat. no. B44170-66)
2. Trizol™ (Gibco-BRL; cat. no. 15596-018).
3. DEPC water: deionized water DEPC treated (0.1% v/v). The DEPC and water were mixed, left standing overnight and autoclaved 30 min. the following day.
4. Agarose (Gibco-BRL; cat. no. 15510-019).
5. 10x TBE agarose buffer: 89 mM Tris, 89 mM boric acid, 2 mM EDTA.
6. Ethidium bromide: 1 mg/ml (Sigma; cat. no. E-7637).
7. Agarose gel loading dye: 10 mM Tris pH 8.0, 20 mM EDTA, 30% glycerol (v/v), 0.5% bromophenol blue (w/v).

2.1.3. Enzymes and buffers

1. T4 Polynucleotide kinase 10 U/µl (Gibco-BRL; cat. no. 18004-028).
2. 5x Forward reaction buffer (provided with T4 Polynucleotide kinase) 0.35 M Tris-HCl, pH 7.6, 50 mM $MgCl_2$, 0.5 M KCl, 5 mM 2-mercaptoethanol.
3. S1 Nuclease 110 U/µl (Amersham; cat. no. E2401Y).

2.1.4. Radionucleotide

1. [γ-^{32}P] ATP (7000 Ci/mmol; ICN, Montréal, QUE, Canada; cat. no. 35020) diluted with DEPC-treated water to give a concentration of 100 µCi/µl on the calibration date of the isotope batch.

2.1.5. Oligonucleotide probe solution hybridization assay

1. Dimethyldichlorosilane solution: 2% dimethyldichlorosilane in 1,1,1-trichloroethane (BDH; cat. no. 331644).
2. Acrylamide and bis-acrylamide (Gibco-BRL; cat. nos. 15512-023, 15516-024).
3. Urea (FisherBiotech; cat. no. BP169-212).
4. 40% (w/v) Acrylamide mix: acrylamide 38% (w/v), bis-acrylamide 2% (w/v). Filter sterilized, degassed and stored at 4°C covered with aluminum foil.
5. Acrylamide top mix: 46% (w/v) urea, 1x TBE sequencing buffer, 8% (w/v) acrylamide mix. Heated gently until the urea was dissolved, filtered, degassed and stored at 4°C.
6. 10x TBE sequencing buffer: 0.89 M tris-borate, 0.89 M boric acid, 25 mM EDTA.
7. APS: 25% (w/v) ammonium persulfate (BDH; cat. no. B10032), freshly made.

8. TEMED: N,N,N',N'-tetramethylethylenediamine (Gibco-BRL; cat. no. 5524UB).
9. 3 M Sodium acetate, pH 5.O: 01.% (v/v) DEPC treated.
10. 70% (v/v) Ethanol: 95% (v/v) ethanol diluted with DEPC water in a sterile 50-ml polypropylene tube. Stored at -20°C.
11. 100% deionized formamide: deionized to bring the pH to 7 by adding 1 g of amberlite monobed resin (BDH; cat. no. B55007) per 10 ml formamide. Stirred for 1 hr at room temperature, filtered through Whatman No. 4 and stored at -20°C in 40 ml aliquots.
12. Sequencing gel loading dye: 95% (v/v) deionized formamide, 20 mM EDTA, pH 8.0, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol in DEPC water.
13. Sequencing gel loading buffer: sequencing gel loading dye without bromophenol blue and xylene cyanol.
14. 0.5 M EDTA, pH 8.0: DEPC treated.
15. 1 M Tris-HCl, pH 8.0. Made up in DEPC water and filter sterilized.
16. TE buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0. Made up in DEPC water using 0.5 M EDTA, pH 8.0, and 1 M Tris-HCl, pH 8.0.
17. Buffered-phenol: 0.1% (w/v) 8-hydroxyquinoline was dissolved in molecular biology grade phenol and an equal volume of 1 M Tris-HCl, pH 8.0 was added. The phenol and tris-HCl were mixed, left standing until the phases separated and the aqueous layer was removed. A second equal volume of 1 M Tris-HCl, pH 8.0 was added and the previous steps repeated. Finally, the phenol phase was topped with 20% (v/v) TE buffer and stored either at 4°C in a dark bottle (working stock) or in 30 ml aliquots in aluminum foil-covered 50-ml polypropylene tubes at -20°C (long-term storage).
18. PCI: buffered phenol, chloroform and isoamyl alcohol mixed in a ratio of 25:24:1 (v/v) and stored as for the buffered phenol working stock.
19. Sephadex® G-50 DNA grade medium (Pharmacia Biotech; cat. no. 17-0043-01) stored in TE pH 7.6.
20. Oligonucleotide solution at 0.03 pmol/µl.
21. Yeast tRNA (100 µg/µl): 100 mg yeast tRNA (Boehringer Mannheim; cat. no. 109517) were dissolved in 1 ml TE buffer containing 0.1 M NaCl. 1 ml buffered-phenol was added, mixed well to extract the proteins then spun at 10,000 g for 10 min. The aqueous layer was removed and extracted again with buffered-phenol followed by two extractions with chloroform. The yeast tRNA was precipitated with 0.1 vol. 3 M sodium acetate, pH 5.O, and 2.5 vol. 95% (v/v) ethanol. After an

incubation on ice for 20 min., the tRNA was recovered by centrifugation at 10,000 g for 30 min. at 4°C. The supernatant was removed, the pellet air dried and resuspended in TE buffer to a concentration of 100 μg/μl and stored in 100 μl aliquots at -20°C.

22. Yeast tRNA (2 μg/μl): a working stock of 2 μg/μl yeast tRNA was prepared by diluting an aliquot of 100 μg/μl yeast tRNA with DEPC water.
23. 50 mM PIPES, pH 6.4: made up in DEPC water. After adjusting the pH with 2 M sodium hydroxide, the solution was brought to the desired volume with DEPC water and filter sterilized.
24. 4 M NaCl: DEPC treated.
25. S1 hybridization buffer: 0.4 M NaCl, 40 mM PIPES, pH 6.4, 1 mM EDTA. Made up in DEPC water using 50 mM PIPES, pH 6.4, 4 M NaCl and 0.5 M EDTA, pH 8.0 in a sterile 50-ml polypropylene tube, filter-sterilized and stored at -80°C.
26. 100 μg/ml Sheared salmon sperm DNA (SSDNA): made up in TE buffer using salmon sperm DNA, type III, sodium salt (Sigma; cat. no. D1626). Sheared by autoclaving for 5 min. and stored in 500 μl aliquots at -20°C.
27. 50 mM Zinc sulfate: DEPC treated.
28. 0.5 M Sodium acetate, pH 4.2: DEPC treated.
29. S1 nuclease buffer: 4.5 mM zinc sulfate, 50 mM sodium acetate, pH 4.2, 0.3 M NaCl. Made up in DEPC water using 50 mM zinc sulfate, 0.5 M sodium acetate, pH 4.2, and 4 M NaCl in a sterile 50-ml polypropylene tube, filter-sterilized and stored at 4°C.
30. Saran wrap.
31. 3M #56 electrical tape (sequencing tape).
32. TLC plates: silica gel thin layer chromatography plate with zinc silicate fluorescent (254 nm) indicator (Fisher Scientific; cat. no. 06-601C).
33. Whatman 3MM filter paper.
34. Model 543 Gel dryer cellophane backing membrane (BIO-RAD; cat. no. 1650922).
35. Biomax film (Kodak, New Haven CT; cat. no. 8715187).

2.1.6. Oligonucleotide probes

All oligonucleotides probes used in the following Chapters were synthesized 'in house' at the Biochemistry Department of the University of Alberta on a PE/ABI DNA synthesizer (model 394-5, Foster City, CA). Table 2.1 below summarizes the subunit transcript specificity and the literature reference for each oligonucleotide probe used.

Subunit transcript specificity **Probe length**	**Oligonucleotide sequence**	**Reference**
α1 45 bases	5'GGGGTCACCCCTGGCTAAGTTAGGGGTATAGCTGGTTGCTGTAGG-3'	(Khrestchatisky et al., 1989) bases 1210-1166.
α 2 33 bases	5'-AGATTCGGGGCGTAGTTGGCAACGGCTACAGCA-3'	(Khrestchatisky et al., 1991) bases 1477-1445.
α 3 50 bases	5'-CTCAGCAGGACTGTCTTGCACATAAGTGGTCTTGGGGGAAGCAATCACTG-3'	(Malherbe et al., 1990) bases 1582-1533.
α 4 30 bases	5'-CAAGTCGCCAGGCACAGGACGTGCAGGAGG-3'	(Wisden et al., 1991) bases (-30)-(-1).
α 5 41 bases	5'-GCTGTACCCACAGCATTCCCAGTCCCGCCTGGAAGCTGCTC-3'	(Khrestchatisky et al., 1989)[*] bases 1343-1303.
α 6 60 bases	5'-CGTTGATGGTAAGATGGGCGTTCTACTGAGGACTTTGCTGGCCTCAGAAGATGGAACGAT-3'	(Lüddens et al., 1990) bases 1200-1141.
β1 45 bases	5'-ATGGCAACCATCACAGGAAAAGAGAGAAGCCCCAAACTCTCTCGA-3'	(Ymer et al., 1989) bases 139-95.
β 2 41 bases	5'-TCGTTCCAGGGCGTTGCGGCCAAAACTATGCCTAGGCAACC-3'	(Ymer et al., 1989) bases 1335-1295.
β 3 55 bases	5'-CTGAATTCCTGGTGTCACCAACGCTGCCTGCAACCTCATTCATTTCATTGTGAAC-3'	(Ymer et al., 1989) bases 1244-1190.

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[*] referred to as α4 subunit sequence.

γ1 50 bases	5’-GCAGTCTTCAAAGCAACAGAAAAAGGTAGCACAGTCTTTGCCCTCCAAGC-3’	(Ymer et al., 1990) bases 1266-1217.
γ2 33 bases	5’-GTTCATTTGGATCGTTGCTGATCTGGGACGGAT-3’	(Shivers et al., 1989) bases 1215-1183
γ3 55 bases	5’-AGAGGGTGCTTGAAGGCTTATTCGATCAGGAATCCATCTTGTTGAATCTGGATGT-3’	(Herb et al., 1992) bases 1215-1170.
β-actin 37 bases	5’-CTGGTGGCGGGTGTGGACCGGGACGGAGGAGCTGCAA-3’	(Nudel et al., 1983) bases 308-272.
GABA-T 65 bases	5’-GATGTATCTGGTACCAGGTGTGAAGAGGTGGAGGTTTTTCTGGGAACTGCAGACCAGCCGTCGGG-3’	(Medina-Kauwe et al., 1994) bases 84-20.
GAT-1 42 bases	5’-GGTAGAGATCTGCCCATCAGCCACCTTGCTGTTGTCAGTCGC-3’	(Guastella et al., 1990) bases 194-153.
GAD$_{65}$ 47 bases	5’-GGCGTCCACACTGCAAGGCCTTGTCTCCCGTGTCATAGGACAGGTCA-3’	(Erlander et al., 1991) bases 1419-1372.
GAD$_{67}$ 55 bases	5’-AGGTTGGTAGTATTAGGATCCGCTCCCGCGTTCGAGGAGGTTGCAGGCGAAGGCG -3’	(Michelsen et al., 1991) bases 252-198.
CCK 45 bases	5’-CTACGATGGGTATTCGTAGTCCTCAGCACTGCGCCGGCCGAAATC-3’	(Deschenes et al., 1985) bases 381-337.
CCK$_{A}$ 55 bases	5’-GCCAGTGCTCGGCTTCTTCTCTTTAGCAGATTTCTTCTGGCTGGCATCAAATTTG-3’	(Takata et al., 1995) bases 14170-14151, 13216-13182.

CCK$_B$ 50 bases	5’-CAAAAGCAGTAGCAGTAGCACGGACCAGGTTTGTTGGACACGTGCACTGG-3’	(Jagerschmidt et al., 1994) bases 889-865, 598-574.
GABA$_B$ 60 bases	5’-TCACTTGTAAAGCAAATGTACTCGACTCCCATCACAGCTAAGCCGGTCAGGGGGCTCAGA-3’	(Kaupmann et al., 1997) bases 2883-2824.

Table 2.1 Oligonucleotide probes
The mRNA species specificity, oligonucleotide size and sequence position within the reference are indicated.

2.2. METHODS

2.2.1. Surgical procedures for drug delivery and animal care

Chronic drug delivery was achieved by the implantation of osmotic minipumps in the dorsal thoracic region under anesthesia. The surgical, animal handling and necropsy procedures were in accordance with the University of Alberta Health Sciences Animal Welfare Committee regulations (protocol numbers 235/04/95 and 235/04/96). Male Sprague-Dawley rats weighing 200 ± 25g upon arrival were housed 2 per cage under a 12/12 hour light/dark cycle. Upon arrival, the animals were kept for 3- 4 days in the animal house before undergoing the surgical procedure to allow for recovery from the shipment. The day preceding the surgery, the animals were weighed and the osmotic minipumps primed overnight at room temperature in saline (sterile NaCl 0.9% v/v) according to the manufacturer’s instructions. Buspirone (4 mg/kg/day), imipramine (20 mg/kg/day) and phenelzine (15 mg/kg/day) were dissolved in water, whereas diazepam (10 mg/kg/day) and alprazolam (10 mg/kg/day) were delivered in a propylene glycol/DMSO (50/50% v/v) vehicle. Since the effects of diazepam on GABA$_A$ receptor subunit gene expression have been well characterized (Marley and Gallager, 1989; Heninger et al., 1990; Primus and Gallager, 1992), a group of animals was treated with this drug in order to test the sensitivity of the multiprobe solution hybridization assay.

The following day rats were implanted with drug- (n = 6) or vehicle- (n = 3) containing osmotic minipumps. Surgical materials (blades, clips, clip applier, clip remover, gauze) were placed in diluted Savlon at the start of the procedure. Rats were anesthetized by inhalation of metophane and then shaved in the interscapular region. The skin was cleaned with Savlon and a 2-3 cm-long incision was made along the dorsal midline of the body at the level of the shoulder blades. A subcutaneous pocket was made by running blunt-end scissors under the skin. After checking that the size and quality of the pocket was adequate, the minipump was rinsed with saline, inserted in the pocket and the wound closed with clips. The rats were placed for recovery under a heating lamp, and the wound was covered with Xilocaine and Eye and Wound Powder. Full recovery from the anesthesia was decided when the animal was moving steadily in the cage under the heating lamp, and only then was the animal was returned to its home cage.

Every subsequent day for the treatment period, animals were examined for wound healing, and the pumps were turned in their subcutaneous pockets. At the end of the treatment period, the rats were decapitated, and the brain quickly removed and dissected on ice. The animals were sacrificed without anesthesia to avoid an interference of the anesthetic on $GABA_A$ receptors (Lin et al., 1993) in order to determine only the effects produced by the drug tested. Tissue samples were frozen in an ethanol/dry ice bath, transferred on dry ice for transport and stored at -80°C until further analysis. The brains were dissected into cortex, cerebellum, brainstem, hippocampus and striatum for mRNA levels analysis, and liver and spinal cord samples were also taken for drug delivery determinations.

2.2.2. RNA isolation from brain samples

Total RNA populations were isolated from the brain samples by using Trizol reagent (Gibco-BRL) according the manufacturer's instructions. The frozen brain samples were added to a corresponding aliquot of Trizol (1 ml/100 mg of tissue), and the tissue was homogenized. The homogenates were incubated at room temperature for 5 min., and chloroform extracted (200 µl/ml of Trizol). After 3 min. incubation at room temperature, the phases were separated by centrifugation at 10,000 g for 15 min. at 4°C and the aqueous phase was transferred to a fresh tube. RNA was precipitated from the aqueous phase by the addition of isopropanol (500 µl/ml of Trizol) and incubation for 10 min. at room temperature. Total RNA was recovered by centrifugation at 10,000 g for 10 min. at 4°C. The pellet was washed once with 1 ml ice-cold 70 % (v/v) ethanol, air-dried and resuspended in DEPC water (1 µl/mg of tissue). The yield and concentration of total RNA recovered were determined by measuring the absorbance at 260 nm (1 A_{260} unit is equivalent to 40 mg/ml of RNA). Protein contamination was determined by the ratio of the A_{260} to A_{280} which is the proportion between the nucleic acid and protein peaks of absorption, respectively. The integrity of the RNA (i.e. degradation) was assessed by running a 10 µg total RNA aliquot mixed with 2 µl agarose gel loading dye and ethidium bromide (final concentration 0.2 mg/ml) through a 0.8% (w/v) agarose gel (2 hr at 70 V with 1x TBE agarose buffer). The visualization of the RNA sample in the gel under ultraviolet lighting should reveal intact 28S and 18S ribosomal RNA bands on a light RNA background smear. RNA samples were stored in 0.3 M sodium acetate, pH 5, 70% ethanol at -80°C until required.

2.2.3. Multiprobe solution hybridization

2.2.3.1. Sequencing gel preparation

A set of glass sequencing electrophoresis plates, spacers and comb were carefully cleaned and assembled as previously described (Tanay et al., 1997; Bateson and Tanay, 1998). Briefly, 8% (w/v) acrylamide sequencing gels (50 ml of 8% (w/v) acrylamide top mix with 70 µl of APS and 70 µl of TEMED) were poured on the days required. Once polymerized, the clips and the bottom tape were removed from the gel-plate assembly, and the latter was rinsed with water to wash off the polymerized acrylamide from the outside of the plates. The gel-plate assembly was mounted onto a sequencing gel electrophoresis apparatus with an aluminum plate placed on the outer side of the assembly in order to maintain an even distribution of the heat over the surface of the gel. Both reservoirs were filled with 1 x TBE sequencing buffer, and flushed with the buffer to eliminate urea from the wells. The gel was pre-run for 1 hr at 33 mA and ready to be loaded when the temperature of the aluminum plate reached 50-55°C.

2.2.3.2. Oligonucleotide purification

The synthesized oligonucleotide probes were purified as described previously (Tanay et al., 1997; Bateson and Tanay, 1998). The oligonucleotide precipitate was resuspended in DEPC water, and the absorbance measured (1 A_{260} unit is equivalent to is 20 µg/ml). An aliquot of the oligonucleotide solution (containing 40 µg) was mixed with an equal volume of sequencing gel loading buffer, heated at 90°C for 5 min. and loaded onto a 8% (w/v) acrylamide sequencing gel. The gel was run for 1 hr at constant current (33 mA). The gel-plate assembly was dismantled and the gel was covered on both sides by pieces of Saran wrap. The Saran wrapped-gel sandwich was taken to a dark room and placed on a fluorescent TLC plate. Under illumination with a short-wave (254 nm) ultraviolet lamp, the position of the nucleotide band of interest was marked on the Saran wrap. The piece of gel was excised with a sterile scalpel blade, separated from the Saran wrap, and transferred to a sterile microfuge tube. DEPC water was added (500 µl) and the gel fragment was mashed with a 1-ml sterile tip attached to a Gilson pipetter. The microfuge tube was then placed on a vertical rotator and the oligonucleotide extracted overnight at room temperature.

After centrifugation at 10,000 g for 5 min. at room temperature, the aqueous phase was removed, transferred to a fresh tube and extracted with 0.5 ml of PCI (1 min. vortex, spin at 10,000 g for 2 min. at room temperature). The aqueous phase was transferred to a fresh tube and the oligonucleotide precipitated with 50 µl of 3 M sodium acetate, pH 5.0, and 1.38 ml of 95% (v/v) ethanol (incubation 30 min. at -20°C, spin at 10,000 g for 30 min. at 4°C). The pellet was washed once with 0.5 ml of ice-cold 70% (v/v) ethanol, air-dried and resuspended in 50 µl of DEPC water. The yield and concentration of oligonucleotide recovered were determined by measuring the absorbance at 260 nm (1 A_{260} unit is equivalent to 20 µg/ml of oligonucleotide, and the average molecular weight of a nucleotide is 330 g/mol.). The oligonucleotide solution was then diluted to 0.03 pmol/µl.

The purity of the oligonucleotide solution was estimated by labeling an aliquot (2 µl) of the 0.03 pmol/µl solution and running it onto a 8% (w/v) acrylamide gel. The labeling reaction mixture (2 µl of purified oligonucleotide at 0.03 pmol/ µl, 100 µCi of [γ-^{32}P] ATP, 1 x forward reaction buffer and 10 units of T4 polynucleotide kinase in a final volume of 20 µl) was incubated at 37°C for 30 min., and the volume of the sample was brought to 100 µl with DEPC water for extraction with PCI (as described above). The top aqueous phase was spun through a G-50 Sephadex spin column (at 700 g for 2 min.) and a 5 µl aliquot mixed with 5 µl of sequencing gel loading dye was loaded onto an 8% (w/v) acrylamide gel (run at 33 mA for 1 hr). The area of the gel between the 2 dye bands was covered with the cellophane backing membrane, the gel was transferred onto 3MM paper, and covered with Saran wrap. The addition of a backing membrane between the gel and the 3MM paper served to prevent the loss of oligonucleotide during the vacuum drying step. The gel sandwich was dried under vacuum in a gel drier for 1 hr at 80°C. The gel was exposed to X-ray films to reveal the presence of any contaminating degradation bands.

2.2.3.3. S1 nuclease protection assay

In separate microfuge tubes, aliquots of oligonucleotide solution at 0.03 pmol/µl were labeled with 100 µCi of [γ-^{32}P] ATP, 1x forward reaction buffer and 10 units of T4 polynucleotide kinase in a final volume of 40 µl (30 min. at 37°C). The reaction volume was increased to 100 µl, PCI extracted and spun through a Sephadex® G-50 medium column (as described above). Each radiolabeled oligonucleotide solution was diluted to

0.006 pmol/μl with DEPC water, and the specific activity determined on 3 μl aliquots. The different specific activities of the oligonucleotide solutions were adjusted empirically with corresponding unlabelled oligonucleotide solutions at 0.006 pmol/μl to result in autoradiographic bands of equivalent intensities. Each aliquot of 10 μg of total RNA was mixed with 0.03 pmol of each oligonucleotide solution and vacuum dried. The dried pellet was resuspended in 30 μl of S1 hybridization buffer, denatured at 90°C for 5 min. The hybridization mixture was incubated overnight at 65°C.

Sheared SSDNA was denatured by boiling for 5 min. The denatured SSDNA was diluted with ice-cold S1 nuclease buffer to a final concentration of 10 μg/ml. S1 nuclease enzyme was added to a final concentration of 120 U/ml, and an aliquot of 300 μl of the S1 nuclease reaction mixture was added to the hybridization mix. The digestion was carried out at 37°C for 15 min. and stopped by transferring 300 μl of the digested sample to a fresh tube containing 300 μl of isopropanol. The undigested oligonucleotide hybrids were precipitated at 10,000 g for 30 min. at -20°C and the pellets were recovered by centrifugation at 10,000 g for 30 min. at 4°C. The pellets were washed with 70% (v/v) ethanol, air dried and resuspended in 6 μl of DEPC water. After the addition of 6 μl of sequencing gel loading dye, the samples were incubated at 90°C for 5 min. and loaded onto an 8% (w/v) acrylamide sequencing gel. The gels was electrophoresed for about 90 min. at 33 mA. As described above, after the electrophoresis the eared plate was lifted from the gel, a cellophane backing membrane was laid on the top of the gel, and the gel transferred onto 3MM paper. After vacuum drying for 1 hr at 80°C, the gel was apposed to Biomax film.

2.2.4. Quantification of protected oligonucleotides

Images of developed autoradiograms were captured on a Hewlett Packard flat-bed scanner (ScanJet 3C) concurrently with a Kodak® gray scale calibration step tablet. Image files were saved in TIFF format and telnet was used to transfer them to a Power Macintosh (6100/66) for analysis by NIH Image v1.56 (Wayne Rasband, RSB, NIMH, NIH, Bethesda, MD). The images were calibrated in optical density versus pixel gray values with the gray scale calibration tablet. Using an NIH Image macro (Gel Plotting Macro) the optical density plot of each electrophoresis lane was drawn. The area under curve was integrated for each peak, and normalized to the area under the curve value of the internal standard present in the same lane. The spectrophotometric measurement of total RNA content is not accurate since it is affected by the absorbance of the RNA, the DNA and to some extent, by the protein content of the sample. Therefore, the relative quantification of changes in mRNA levels required choosing an internal standard as an indicator of the amount of RNA really present within the samples. House-keeping genes encode proteins required for general cellular functions and are therefore often assumed to be expressed at constant levels between cell types. House-keeping genes are these encoding cytoskeleton microfilament proteins which are involved in the maintenance of the cell structure, attachment of the cell to a substrate, cell mobility and cell division (Becker and Deamer, 1991). Indeed, β-actin is a protein constituent of the cytoskeleton microfilaments present at constant levels throughout the non-muscular vertebrate cells (Becker and Deamer, 1991). β-actin has been used as internal standard by many groups investigating changes in gene expression induced by various conditions (Heninger et al., 1990; Baumgartner et al., 1994; Mhatre and Ticku, 1994b; Zhao et al., 1995), and their results indicate that β-actin gene expression is not altered by chronic drug treatment (Heninger et al., 1990; Mhatre and Ticku, 1994b; Zhao et al., 1995). Regarding the experiments performed for this dissertation, the fact that for each autoradiogram the integrated band intensity for β-actin remained constant between groups, and that changes in expression were observed for a limited number of genes further support that β-actin gene expression remained constant. Thus, β-actin mRNA, which represents approximately 0.003% of total RNA, was chosen as the internal standard. The comparison of the level of low abundance mRNA to that of β-actin presents less inherent error than the comparison to an abundant RNA species such as ribosomal RNA. The normalized value of each oligonucleotide band was expressed as a percentage of the mean standardized value of the vehicle-treated group. The experiments were repeated 2 to 5 times when possible, and the percentage values obtained for each sample were pooled.

2.2.5. Statistical analysis and differences considerations

After pooling the vehicle-treated results from the different groups, statistical analysis was run on the pooled data by Dunnett's test (pairwise comparison of different treatment group with a control group). The level of significance was set for a probability (*P*) of 0.05 or less. The statistical analysis by Dunnett's pairwise comparison to a vehicle group was chosen for two main reasons. Firstly, the vehicle groups were pooled in order to get a better measure of the variance of the vehicle-treated group and to increase the number of degrees of freedom for the statistical analysis. Consequently, multiple Student *t*-test comparisons between the different drug- and the vehicle-treated groups were rendered inappropriate as they increase dramatically the risk of determination of significance by chance. Secondly, the calculation of the *t* critical value for Dunnett's test is based on the mean square error obtained by running a one-way ANOVA over each set of data. Dunnett's comparison is not a "traditional" post-hoc test, in that there is no requirement for a significant *F*-value from the ANOVA to administer this test. The ANOVA calculation provides with the mean square error term that is used to calculate Dunnett's critical value. Dunnett's analysis, like many multiple planned comparison tests, takes into account the number of comparisons to be made, and corrects the error term accordingly. Such a test avoids the disadvantages pertaining to the use of an *F*-test, which is an average test of a set of independent comparisons, if only some of the comparisons include a significant difference. In such a case, this difference is averaged with a number of non-significant differences, and the overall *F*-test may be non-significant. However, if the difference is present for a set of planned comparison, Dunnett's comparison will likely be significant. Consequently, the risk of false determination of significance due to an outstanding low variance of a group, or conversely, the danger of a false negative result due to an unusually large group variance is reduced.

Besides the determination of significant changes in mRNA levels compared to the vehicle-treated group, the central goal of this thesis is to determine whether antipanic drugs produce similar changes in gene expression that are not shared with buspirone. Consequently, in parallel to the statistical calculations, a qualitative analysis was conducted. This qualitative assessment of effects relied on the search for effects produced by antipanic agents that were not shared with buspirone but that were common to the antipanic drugs (same direction of changes, similar amplitude if possible). Changes in subunit gene expression were considered common to the antipanic drugs, when these agents induced changes of similar amplitude whereas buspirone produced no or opposite effects, and for at least two of the three antipanic drugs tested this effect was reflected by a statistically significant difference.

2.2.6. Drug delivery measurements

The drug delivery assays were performed by Mrs. G. Rauw, Department of Psychiatry.

2.2.6.1. Alprazolam and buspirone

The deliveries of buspirone and alprazolam were assessed by measuring their levels in the liver according to a method recently developed (Lai et al., 1997). Livers from treated animals were homogenized in 5 volumes of deionized water (w/v). To 2 ml of homogenate, 1 μg of internal standard (buspirone for alprazolam assay, and alprazolam for buspirone assay) was added and the mixture was basified with 400 μl of 0.61 M Na_3PO_4 (pH 11.5-12) and vortexed. The mixture was extracted with 5 ml of toluene by shaking for 5 min., then centrifugating at 1,000 g for 10 min. at room temperature. The toluene layers were transferred into fresh 13 x 100 mm glass test tubes and dried in a SpeedVac• SC110 evaporator (Savant Instruments, Farmingdale, NY, USA). The dried extracts were reconstituted in 200 μl of glass distilled toluene, and 2 μl was used for gas chromatography. The quantification of the amount of alprazolam or buspirone present within the tissues was obtained by extrapolating the drug/internal standard peak height ratio from calibration curves generated on the day of the assay. Representative chromatograms and a calibration curve for alprazolam and buspirone are presented in Figures 2.1 and 2.2, respectively. The gas chromatography conditions were as follows: Hewlett-Packard 5980 gas chromatograph equipped with a nitrogen-phosphorus detector, Hewlett-Packard 6890 splitless injector with purge off time of 0.5 min., and a cross-linked fused silica capillary column (15 m x 0.25 mm internal diameter) coated with a 0.25 μm film thickness of 5% (v/v) phenylmethylpolysiloxane (DB5, J&W Scientific Inc., Folsom, CA, USA). The oven conditions were as follows: the initial temperature of 105°C was maintained for 0.5 min., then increased at a rate of 15°C/min. to 295°C and maintained for 15 min. The injection port and detector temperatures were 270°C and 325°C respectively, with ultra-pure helium (Praxair Inc., Mississauga, ON, Canada) as the carrier gas (2 ml/min.) and make-up gas (30 ml/min.). The detector was purged with pure hydrogen at 3.5 ml/min. mixed with dry air at 80 ml/min. (Praxair Inc., Mississauga, ON, Canada).

2.2.6.2. Imipramine

The delivery of imipramine was assessed by the measurement of imipramine and its primary active metabolite desipramine in spinal cord by gas chromatography with nitrogen-phosphorus detection (Drebit et al., 1988). Spinal cords were homogenized in 5 volumes of deionized water (w/v), and 500 ng of internal standard (maprotiline) were added to 1 ml of homogenate. The solution was basified with 250 μl of 1.81 M K_2CO_3 (w/v pH 11-11.5), extracted with 4 ml of toluene (as described above), and centrifuged (5 min.) as described above. The toluene layers were removed to clean 16 x 150 mm glass test tubes and dried under nitrogen in boiling water. The residues were reconstituted in 2 ml of double distilled water, with $NaHCO_3$ added to saturation and vortexed. After the addition of 300 μl of acetic anhydride, the acetylation reaction was allowed to proceed until the bubbling ceased and a small amount of $NaHCO_3$ was left. The solutions were transferred into clean 16 x 150 mm tubes and 4 ml of ethyl acetate were added to each. The samples were extracted as described above (shaken and centrifuged for 5 min.). The ethyl acetate layer was transferred to 13 x 100 mm tubes and dried under nitrogen. Finally the residues were reconstituted in 100 µl of toluene and 2 µl was used for gas chromatography. The amount of imipramine and desipramine present within the tissues was quantified by extrapolating the drug/internal standard peak height ratio from calibration curves generated on the day of the assay. Representative peaks and a calibration curve are presented in Figure 2.3. The gas chromatography conditions were as follows: Hewlett-Packard 5980 gas chromatograph equipped with a nitrogen-phosphorus detector and a splitless injector, and a cross-linked fused silica capillary column (15 m x 0.25 mm internal diameter) coated with a 0.25 µm film thickness of 5% phenylmethylpolysiloxane (DB5, J&W Scientific Inc., Folsom, CA, USA). The following are the oven conditions that were used: the initial oven temperature of 105°C was increased at a rate of 12°C/min. to 295°C and maintained at this temperature for 15 min. The injection port and detector temperatures were 270°C and 325°C respectively, with ultra-pure helium (Praxair Inc., Mississauga, ON, Canada) as carrier gas (2 ml/min.) and make-up gas (30 ml/min.). The detector was purged with pure hydrogen at 3.5 ml/min. mixed with dry air at 80 ml/min. (Praxair Inc., Mississauga, ON, Canada).

2.2.6.3. Phenelzine

The delivery of phenelzine was assessed by the determination of monoamine oxidase (MAO) activity and the levels of GABA in the spinal cord. These measurements were performed to ensure that phenelzine drug level was high enough to completely block the activity of both types of monoamine oxidase (which differ in their substrate specificity) and to quantify the increase in GABA levels induced by phenelzine. The determination of GABA levels in the spinal cord was performed by gas chromatography with electron-capture detection (Wong et al., 1990). Spinal cords were homogenized in 5 volumes of ice-cold 0.1 N $HClO_4$ containing 0.05 mM ascorbic acid and 2.7 M EDTA, and centrifuged at 4°C (10 min. at 12,000 g). To 25 µl of the supernatant, 250 ng of norleucine (internal standard in 0.5 M HCl) were added, along with 1 ml of 0.18 M K_2CO_3 and 1 ml of a isobutylchloroformate solution (5 ml/ml in toluene:acetonitrile 9:1, v/v). The samples were extracted (10 min. shake followed by a centrifugation at 1,000 g for 2 min. at room temperature). The organic phase was discarded and 1.5 ml of 2 M NaH_2PO_4 (pH 5.6) and 25 µl of 6 N HCl were added to the aqueous phase. After briefly vortexing, 2.5 ml of chloroform, 200 μl of dicyclohexylcarbodiimide solution (dicyclohexylcarbodiimide: chloroform 5:1, v/v), and 200 μl of pentafluorophenol solution (pentafluorophenol: chloroform 5:1, v/v) were sequentially added and the samples were extracted by vortexing for 15 min. followed by a centrifugation at 1,000 g for 5 min. The aqueous phase was aspirated and the remaining chloroform layer was decanted into 13 x 100 mm tubes. The organic phase was dried under nitrogen and the residues were reconstituted in 300 μl of distilled-in-glass toluene which was washed briefly with 0.5 ml of water and centrifuged at 1,000 g. A 1 μl aliquot of the toluene layer was used for gas chromatography analysis. The quantification of the amount of GABA present within the spinal cord was achieved by extrapolating the GABA/internal standard peak height ratio from calibration curves generated on the day of the assay. Representative peaks and calibration curves are presented in Figure 2.4. The gas chromatography conditions were as follows: Hewlett-Packard 5980A gas chromatograph equipped with a electron-capture detector, Hewlett-Packard 7673A injector with purge off time of 0.5 min., and a fused silica capillary column (25 m x 0.32 mm I.D.) coated with 0.52 μm film thickness of 590 phenyl methyl silicone (Hewlett-Packard Co.). The optimal conditions required an initial oven temperature 100°C maintained for 0.5 min., followed by a temperature increase at a rate of 25°C/min. to 200°C where it was maintained for 0.5 min. The temperature was ramped again at a rate of 3°C/min. to 230°C (held for 0.5 min.), and finally increased at a rate of 25°C/min. to 300°C and maintained at 300°C for 5 min. The injection port and detector temperatures were 200°C and 325°C respectively, with helium at 3 ml/min. as carrier gas, and argon/methane, 30 ml/min., as make-up gas.

MAO activities were determined by a radiochemical method (Lyles and Callingham, 1982). Spinal cords were homogenized in 5 volumes of distilled water. The homogenates were diluted 16 times in 0.2 M KH_2PO_4 (pH 7.8). In 12 x 100 mm culture tubes placed in an ice water bath, 50 μl of the 1:100 dilution of the appropriate radiolabeled substrate (MAO-A activity assay: [^{3}H]- or [^{14}C]-serotonin at 50 mM, 1 mCi/mmol; MAO-B activity assay: [^{14}C]-phenylethylamine at 10 mM, 1 mCi/mmol) was added to 50 μl of tissue homogenate. The samples were flushed with oxygen and quickly capped. The reaction mixture was incubated at 37°C for 10 min. and the tubes placed on ice. Ten microliters of 3 M HCl and 1 ml of ethyl acetate:toluene (1:1 v/v water saturated) were added and the samples were extracted as described above and centrifuged at 400 g for 30 sec. An aliquot of the organic phase (700 μl) was transferred to scintillation vials containing 4.5 ml of scintillation liquid (Ready Safe) and counted for 3 min. The results were expressed as a percentage of inhibition compared to the mean control values. The percentage inhibition was calculated as: 100 - [100 x (sample-blank)/(control- blank)].

The results of the assessment of the different drug delivery parameters for the animal samples used in the multiple probe solution hybridization assay are summarized in Table 2.2 below.

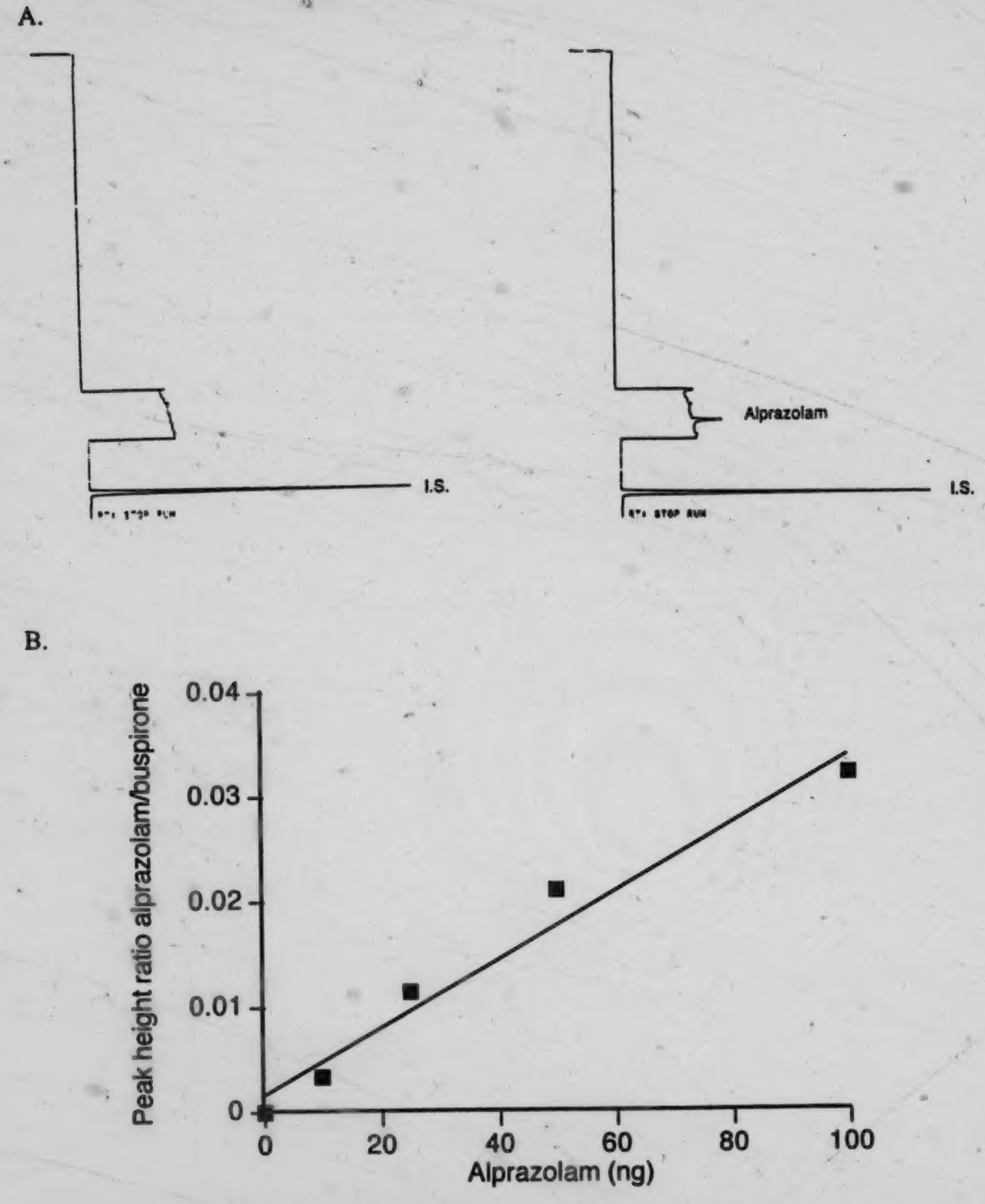

Figure 2.1 Representative chromatogram peaks (A) and calibration curve (B) for the assay of alprazolam levels in liver
Panel A shows representative chromatograms traces of extracted liver homogenates of a vehicle-treated (left trace), or a drug-treated animal (right trace) both spiked with internal standard (I.S.). Panel B presents a representative calibration curve for alprazolam measurement (r^2=0.9684).

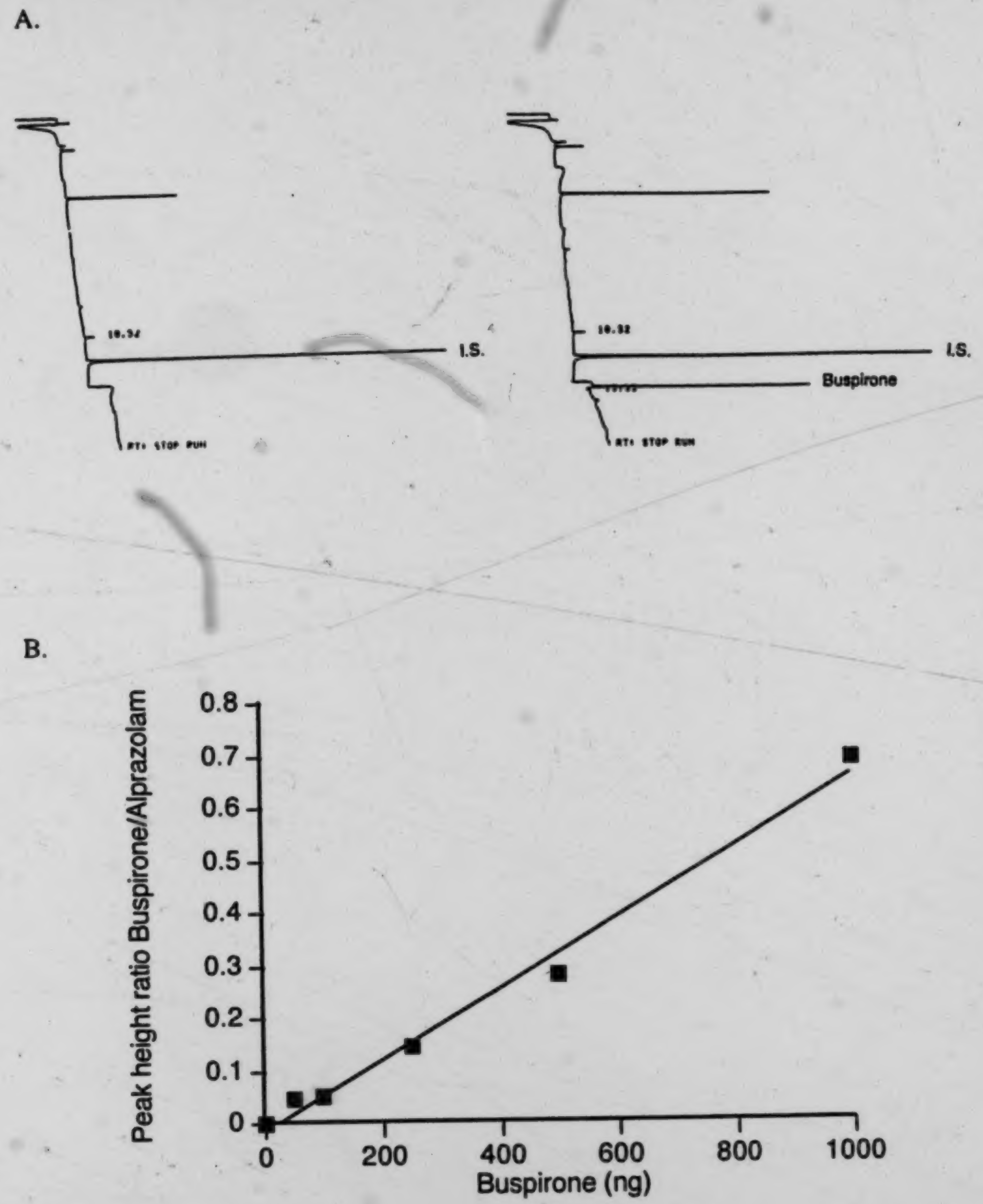

Figure 2.2 Representative chromatogram peaks (A) and calibration curve (B) for the assay of buspirone levels in liver
Panel A shows representative chromatograms traces of extracted liver homogenates of a vehicle-treated (left trace), or a drug-treated animal (right trace) both spiked with internal standard (I.S.). Panel B presents a representative calibration curve for buspirone measurement (r^2=0.9885).

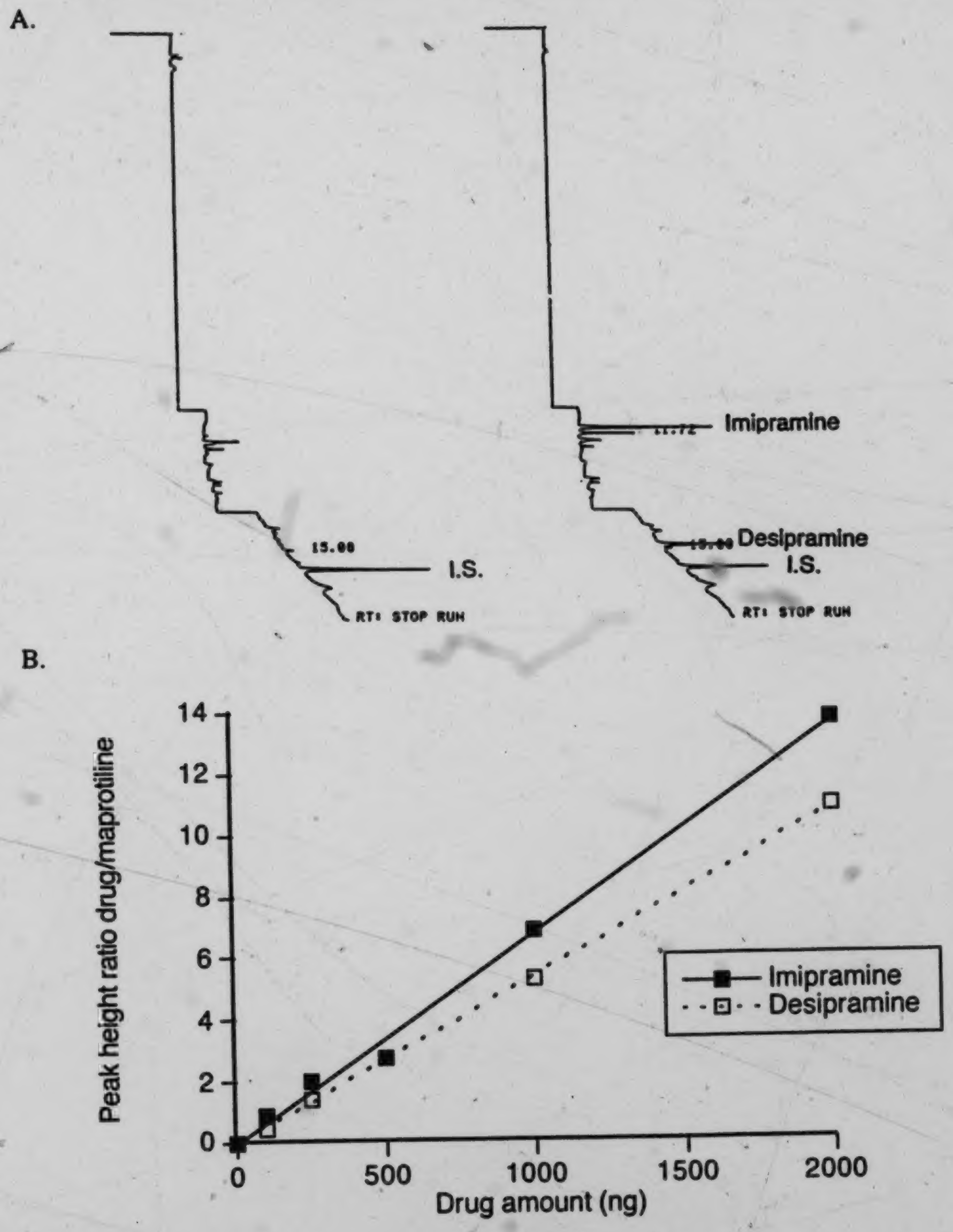

Figure 2.3 Representative chromatogram peaks (A) and calibration curve (B) for the assay of imipramine and desipramine levels in spinal cord
Panel A shows representative chromatograms traces of extracted liver homogenates of a vehicle-treated (left trace), or a drug-treated animal (right trace) both spiked with internal standard (I.S.). Panel B presents representative calibration curves for imipramine (r^2=0.9958) and desipramine (r^2=0.9998) measurement.

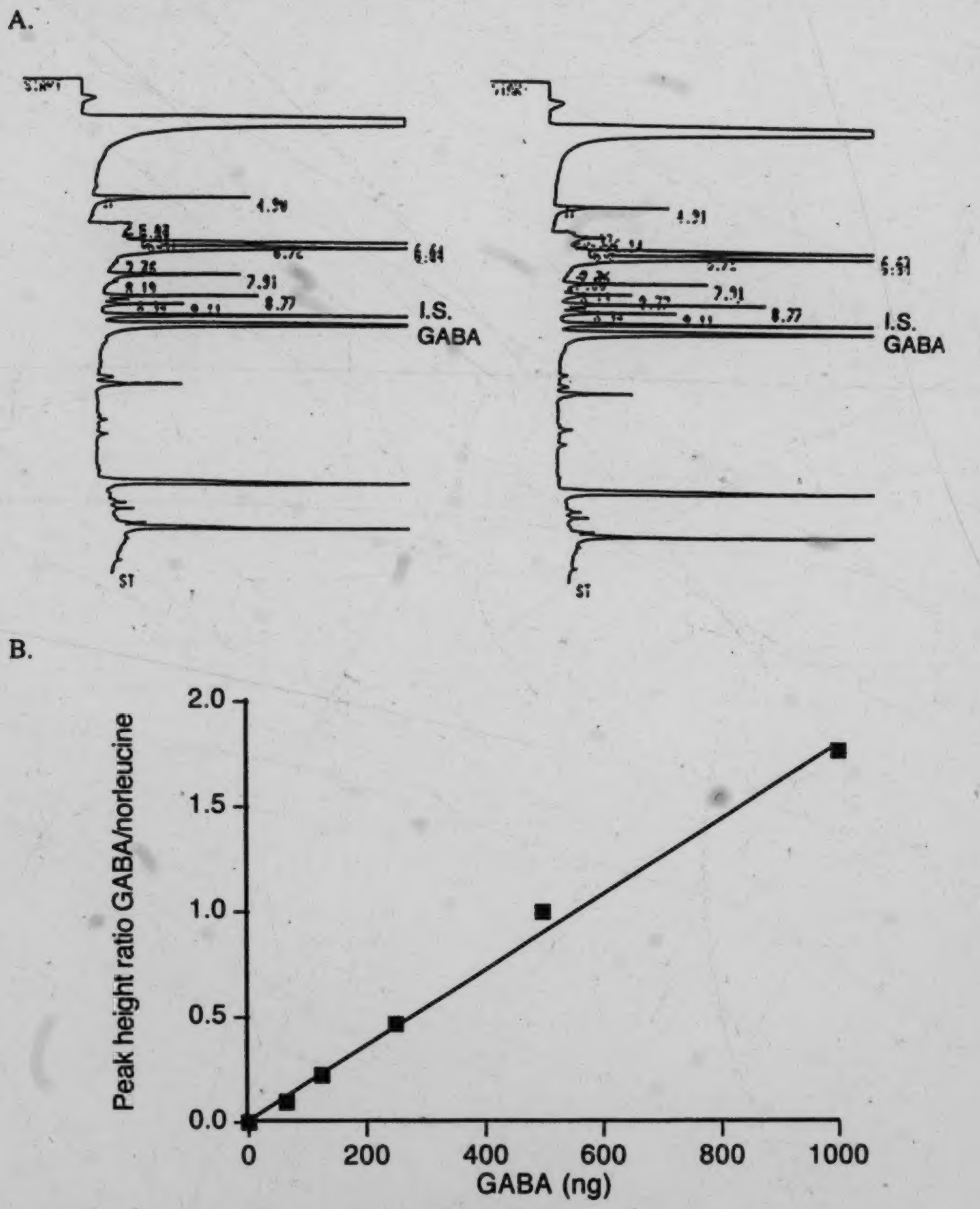

Figure 2.4 Representative chromatogram peaks (A) and calibration curve (B) for the assay of GABA levels in spinal cord
Panel A shows representative chromatograms traces of extracted liver homogenates of a vehicle-treated (left trace), or a drug-treated animal (right trace) both spiked with internal standard (I.S.). Panel B presents a representative calibration curve for GABA measurement (r^2=0.9957).

Treatment duration	**Drug and dosage**	**Assessment of drug delivery**
21 days*	Alprazolam (10 mg/kg/day)	37.44 ± 16.25 ng/g
21 days**	Alprazolam (10 mg/kg/day)	23.37 ± 3.87 ng/g
21 days	Imipramine (20 mg/kg/day)	Imi: 1609 ± 278 ng/g Dmi: 1153± 169 ng/g
21 days	Phenelzine (15 mg/kg/day)	GABA 195 ± 13 % vehicle MAO-A 100 ± 0.06 % inhibition MAO-B 96 ± 0.6 % inhibition
21 days	Buspirone (4 mg/kg/day)	327.7 ± 33.2 ng/g
14 days	Alprazolam (10 mg/kg/day)	92 ± 61.4 ng/g
3 days	Alprazolam (10 mg/kg/day)	32.21 ± 18.08 ng/g
3 days	Imipramine (20 mg/kg/day)	Imi: 1823 ± 261 ng/g Dmi: 1462± 233 ng/g
3 days	Phenelzine (15 mg/kg/day)	GABA 225 ± 13 % vehicle MAO-A 99 ± 1.53 % inhibition MAO-B 101 ± 0.2 % inhibition
3 days	Buspirone (4 mg/kg/day)	287.6 ± 34.7 ng/g

Table 2.2 Assessment of drug delivery in the animal groups used in the multiprobe solution hybridization assay
Drug delivery by the osmotic minipumps was determined according to the protocols described in the text. Imi and Dmi are imipramine and desipramine spinal cord tissue concentrations, respectively. GABA spinal cord tissue levels are reported as a percentage of the mean control group levels, and MAO-A and MAO-B results are expressed as percentage inhibition compared to controls. Alprazolam and buspirone concentrations indicated are from liver samples.

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[*] Group of animals used to generate the cortex and cerebellum data.

[**] Group of animals used to generate the brainstem data.

3. Effect of treatment with antipanic drugs on the gene expression of GABA_A receptor subunits, glutamic acid decarboxylase 65 and 67, GABA transporter-1 and GABA transaminase in the cortex [*]

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[*] A portion of this chapter has been published: (Lai et al., 1998).

3.1. INTRODUCTION

The effects of chronic antipanic drug treatment on aspects of GABAergic transmission were first investigated in cortex. The cortex receives extensive input from external sensory pathways and the lower brain regions such as the brainstem LC and raphé nuclei (Rouiller et al., 1989). Evidence supports a cognitive model of PD which suggests that patients may be prone to misinterpret bodily sensations (Kamieniecki et al., 1997) and display exaggerated stress responses to mild psychological stressors (Leyton et al., 1996). Further, a neuroanatomical hypothesis of PD suggests that the phobic avoidance which develops in PD patients is related to the activation of cortical areas (Rosenberg, 1993; Gorman et al., 1989). These hypotheses may provide a basis for the relative efficacy of cognitive therapy in PD (Black et al., 1993; Marks et al., 1993). In addition, cortical GABAergic pathways are ubiquitous and thought to be of importance for the regulation of the somatosensory inputs to the cortex (Barnstable et al., 1992; Berman et al., 1992; Ebner and Armstrong-James, 1990), suggesting that this neurotransmitter may be involved in the modulation of cortical functions. Finally, imaging studies suggest that a decreased cortical benzodiazepine binding site density may be present in PD patients (Kuikka et al., 1995; Schlegel et al., 1994; Malizia et al., 1997). Hence, it is possible that aspects of PD may be linked to an abnormal GABAergic transmission in the cortex, and that part of the antipanic efficacy of drugs might be related to changes in $GABA_A$ receptor subunit gene expression in the cortex.

In parallel to the determination of alterations in $GABA_A$ receptor subunit gene expression, other aspects of the GABAergic transmission were investigated in the cortex of imipramine- and phenelzine-treated rats. As previously mentioned, the effect of phenelzine on brain GABA levels are due, at least in part, to the inhibition of GABA-T by phenelzine (McKenna et al., 1994; McManus et al., 1992; Popov and Matthies, 1969). This inhibition of GABA-T may be mediated by the reduction of plasma vitamin B6 (a cofactor for both GABA-T and GAD) which has been observed in patients taking phenelzine (Malcolm et al., 1994). In addition, recent evidence indicates that phenelzine augments extracellular GABA levels (Parent et al., 1998). In contrast, imipramine which was reported to increase GABA release from rat thalamus (Korf and Venema, 1983), did not alter GABA concentrations in rat cortex (McManus et al., 1992). Brain GABA turnover involves three separate steps: synthesis by the enzyme GAD, degradation by the enzyme GABA-T, and the GABA-transporter (GAT) which removes GABA from the synaptic cleft (Kugler, 1993). The rate-limiting step in GABA metabolism is the synthesis by GAD of GABA from glutamate (Kugler, 1993). Two forms of GAD, each encoded by a separate gene, have been identified which are isoenzymes of 65 kDa and 67 kDa denoted GAD_{65} and GAD_{67}, respectively (Erlander et al., 1991). GABA-T, which is involved in the catabolism of GABA, requires pyridoxal 5'-phosphate as a cofactor for its metabolic activity and is found in both neurons and glial cells (Kugler, 1993). The heterogeneity of rat GAT has been unveiled by the cloning of 3 subtypes (GAT-1, GAT-2 and GAT-3) (Borden et al., 1992; Guastella et al., 1990; Clark et al., 1992). Whereas GAT-1 and GAT-3 are expressed at high levels in the brain, GAT-2 brain expression levels are low, but this transporter subtype is also found in the periphery (Ikegaki et al., 1994; Borden et al., 1992). GAT-1 is found in the cortex (Ikegaki et al., 1994), and evidence suggests that the distribution of this transporter subtype involves mainly GABAergic neurons, but it is also found in astroglia and some non-GABAergic neuronal cells (Rattray and Priestley, 1993; Swan et al., 1994; Minelli et al., 1995; Yasumi et al., 1997). In contrast, GAT-3 is present exclusively on astrocytic processes of glial cells in the cortex (Minelli et al., 1996) and in the cerebellum (Itouji et al., 1996) and therefore is speculated to be responsible for GABA reuptake in glial cells (Itouji et al., 1996; Minelli et al., 1996). The differential distribution between GAT-1 and GAT-3 suggest that these transporters regulate different aspects of GABA-mediated transmission.

The size of the cortical tissue sample and relative ease with which large amounts of total RNA can be obtained make it a region of choice for conducting experiments testing the sensitivity of the multiprobe solution hybridization technique. A test of this technique was performed by including in the experiments a drug, diazepam, for which effects on $GABA_A$ receptor subunit gene expression have been well documented (Primus and Gallager, 1992; Heninger et al., 1990; Marley and Gallager, 1989). In addition, recent data indicate that diazepam may possess antipanic properties (Noyes et al., 1996).

After validating the multiprobe solution hybridization technique by establishing the sensitivity of the detection method and the range of linearity for the conditions used, the effects of chronic diazepam treatment on $GABA_A$ receptor subunit transcript levels were determined in order to verify that the experimental design used could replicate previous findings. Therefore, $GABA_A$ receptor subunit steady-state mRNA levels were measured in animals chronically treated with either: buspirone, diazepam or one of the antipanic drugs. These experiments also aimed at unveiling any possible targeting of $GABA_A$ receptor subunit gene expression common to the antipanic drugs.

Finally, the chronic effects of phenelzine and imipramine on GABA levels, led to the investigation of whether: (1) the effects of phenelzine on brain GABA levels would trigger compensatory mechanisms (such as increases in transport or catabolism) to counteract the increase in this neurotransmitter concentrations; and (2) the effects of imipramine on GABA release would be associated with alterations in GABA metabolism or transport. The parameters investigated included the study of alteration in gene expression of the GABA metabolizing enzyme, GABA-T, the rate-limiting GABA synthesizing enzymes, GAD_{65} and GAD_{67}, and the most abundant and widespread GABA transporter in cortex, GAT-1. These chronic effects at 21 days of treatment were compared to the effects produced after an acute treatment of 3 days. For each time point, the mRNA levels of the treated animals were expressed as percentage variation compared to their respective controls. The vehicle groups were pooled by treatment period for statistical analysis with Dunnett's pairwise comparison.

3.2. RESULTS

3.2.1. Method validation

The linearity range of the detection procedure (autoradiographic film and densitometric analysis of the autoradiogram bands) was investigated by establishing the relationship between the amount of radioactivity contained within the gel bands and the optical density of the corresponding autoradiogram bands within the same exposed film. Serial dilutions of radiolabeled oligonucleotides were separated by electrophoresis and the autoradiographic images of the gel lanes were obtained. The optical densities of the autoradiographic bands were plotted against the amount of radioactivity loaded onto the gel (Figure 3.1). The relationship between the amount of radioactivity present within the gel and the optical density of the resulting bands was established to be linear for integrated band optical intensities ranging from 0 to 6,000 (regression analysis for β-actin specific oligonucleotide was $r^2 = 0.9961$, $p<0.05$).

The multiprobe oligonucleotide solution hybridization technique relies on the assumption that each molecule of a target mRNA species is hybridized to a corresponding oligonucleotide probe molecule. Therefore, the oligonucleotide probes need to be present in excess and the hybridization reaction must be allowed to proceed to completion. As such, an increase in the amount of mRNA species present should result in a proportional increase in the optical density of the bands (Figure [illegible].2). The relationship between the amount of total cortex RNA and the optical density was linear over the range of 0-20 μg (regression analysis for β-actin specific oligonucleotide $r^2 = 0.9899$, $p<0.05$).

3.2.2. Effects of chronic drug treatments on GABA$_A$ receptor subunit gene expression

The pattern of changes in α-subunit gene expression was drug specific (Figure 3.3). The levels of α6- and α4-subunit mRNA could not be reliably quantified in the imipramine and phenelzine-treated samples (respectively), since the integrated band optical intensities corresponding to these subunits were below 20 arbitrary units in many experimental replicates. These data points were therefore determined to be unreliable and excluded from the pooled data (see section 3.3). Buspirone was the only drug which induced a significant increase (128%) in α2-subunit gene expression. Overall, buspirone seemed to increase α-subunit mRNA levels, while alprazolam produced increases in α2- (61%) and α4-subunit (46%) gene expression that failed to reach significance. In contrast, neither imipramine, phenelzine nor diazepam seem to dramatically affect the expression of these subunits (the changes were less than 40%).

In the same manner as for the α-subunits, the pattern of changes in the β-subunit steady-state mRNA levels was drug specific (Figure 3.4). Again, only a few statistically significant changes were found, such as the effect of phenelzine on β1-subunit (93% increase) mRNA levels. Although alprazolam and buspirone also increased the levels of β1-subunit (56% and 67% respectively), these effects were not significant. Overall, alprazolam, buspirone and phenelzine seemed to increase β-subunit gene expression, whereas diazepam and imipramine did not markedly affect GABA$_A$ receptor β-subunit gene expression (changes less than 40%).

The alterations in γ-subunit gene expression after chronic treatment with the various drugs are presented in Figure 3.5. Similar to the α- and β-subunits, the changes in γ-subunit mRNA levels were drug specific, but a greater number of significant variations were found. Alprazolam and phenelzine significantly induced the expression of the γ3-subunit gene (75% and 70%, respectively), and buspirone increased both the expression of γ1- and γ3-subunit genes (183% and 112%, respectively). Imipramine affected the expression of γ1- and γ3-subunit genes (48% and 42% increase, respectively) but these alterations were not significant. Alprazolam, buspirone and phenelzine seemed to noticeably increase the expression of γ-subunit genes, whereas imipramine presented a weaker effect and diazepam had no effect.

3.2.3. Effects of phenelzine or imipramine treatments on GABA-T, GAD_{65} and GAD_{67}, and GAT-1

The data presented in this section are the result of collaborative work with Dr. C.T. Lai who performed the solution hybridization assays on total RNA samples that I had prepared beforehand from the groups of animals which were treated with imipramine or phenelzine. The data analysis was a collaboration between Dr. C.T. Lai and myself. The data published were extracted from one experiment (n=6) (Lai et al., 1998), whereas the data presented here represents the pooled results from either 2 (imipramine 3 day and 21 day values), or 3 (phenelzine 21 day values), or 5 separate assays (phenelzine 3 day values) with an n of 6 per experiment.

After 3 days of treatment, neither phenelzine nor imipramine induced changes in the expression levels of GAD_{65} and GAD_{67}, GABA-T, or GAT-1 (Figure 3.6 panel A). After 21 days of treatment, imipramine still had no effects (Figure 3.6 panel B). However, the steady state mRNA levels encoding for GABA-T and the GAT-1 were significantly increased by 15% following chronic treatment with phenelzine (Figure 3.6 panel B).

3.2.4. Effects of alprazolam, buspirone or phenelzine on $GABA_A$ receptor subunit gene cluster expression

The changes in $GABA_A$ receptor subunit gene expression grouped by gene cluster are presented in Figure 3.7. This diagram revealed that the scatter of the effects induced by chronic phenelzine treatment was large, and that the mean alterations in subunit mRNA levels were distributed on each side of the 0% variation for the 3 gene clusters. In contrast, both alprazolam and buspirone displayed a smaller distribution of effect on the $\alpha6\alpha1\beta2\gamma2$ subunit gene cluster, and a coordinated increasing effect on the expression of the $\alpha2\alpha4\beta1\gamma1$ gene cluster.

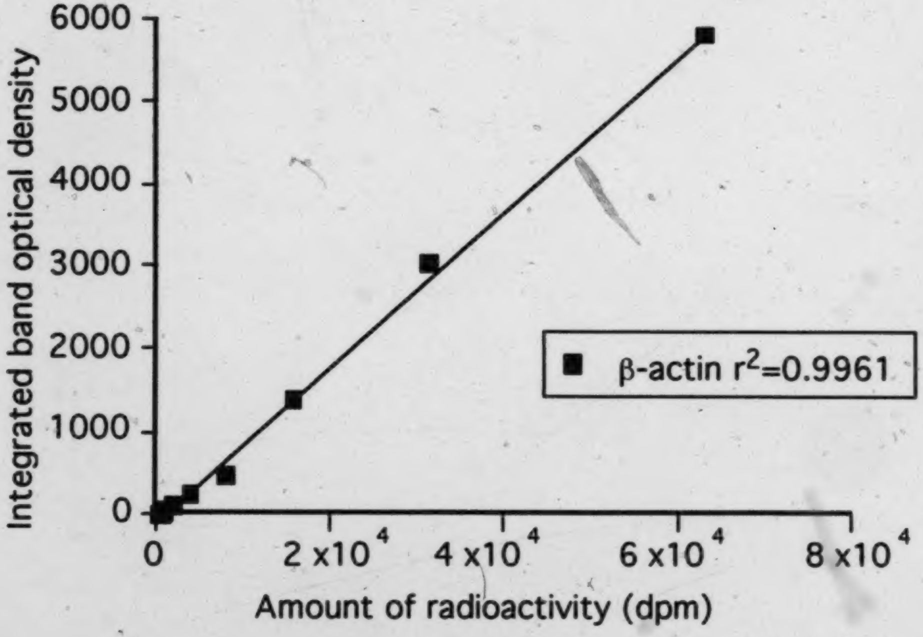

Figure 3.1 Linearity of the detection procedure
Serial dilutions of radiolabeled β-actin specific oligonucleotides were loaded onto an 8% (w/v) acrylamide gel. The integrated optical density of the resulting autoradiographic bands from the same gel exposure were plotted against the amount of radioactivity loaded in each lane. The significance ($P<0.05$) of the correlation was determined by ANOVA regression analysis.

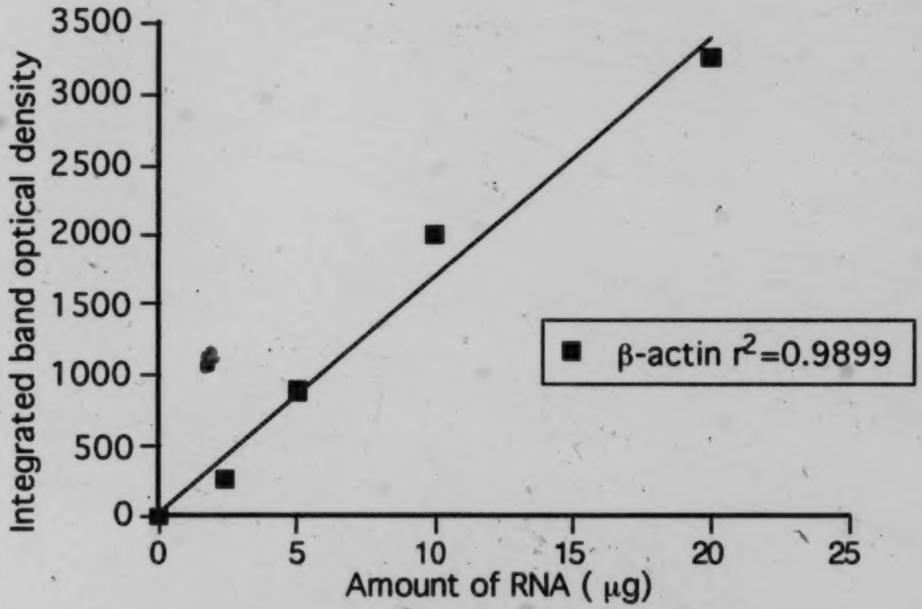

Figure 3.2 Linearity of the multiprobe solution hybridization assay
Multiprobe solution hybridization assays were carried out with a constant β-actin-specific oligonucleotide specific activity, in the presence of increasing amount of total cortex RNA. The resulting optical densities of the autoradiographic bands were integrated and plotted against the corresponding amount of total RNA. The significance ($P<0.05$) of the correlation between the 2 variables was determined by ANOVA regression.

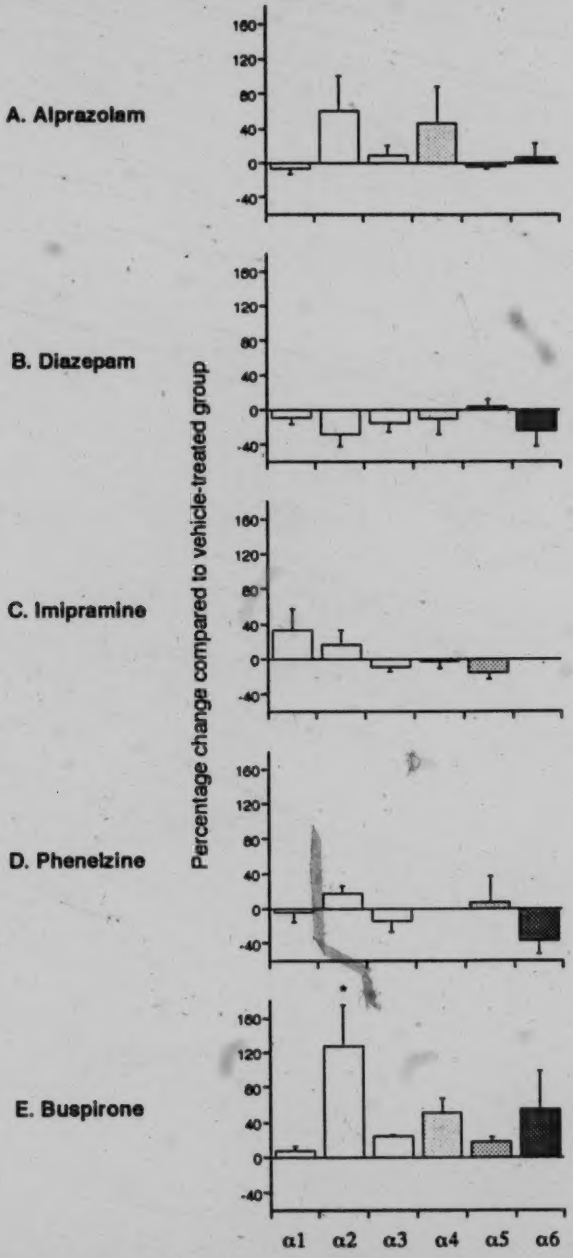

Figure 3.3 Effect of 21 days of drug treatment on cortex α-subunit gene expression
Data represent the mean ± s.e.m. (n=6) percentage change in the various α-subunit mRNA levels relative to the vehicle-treated group. Statistical analyses were performed by the Dunnett's test pairwise comparison between drug- and vehicle-treated groups, * $P < 0.05$.

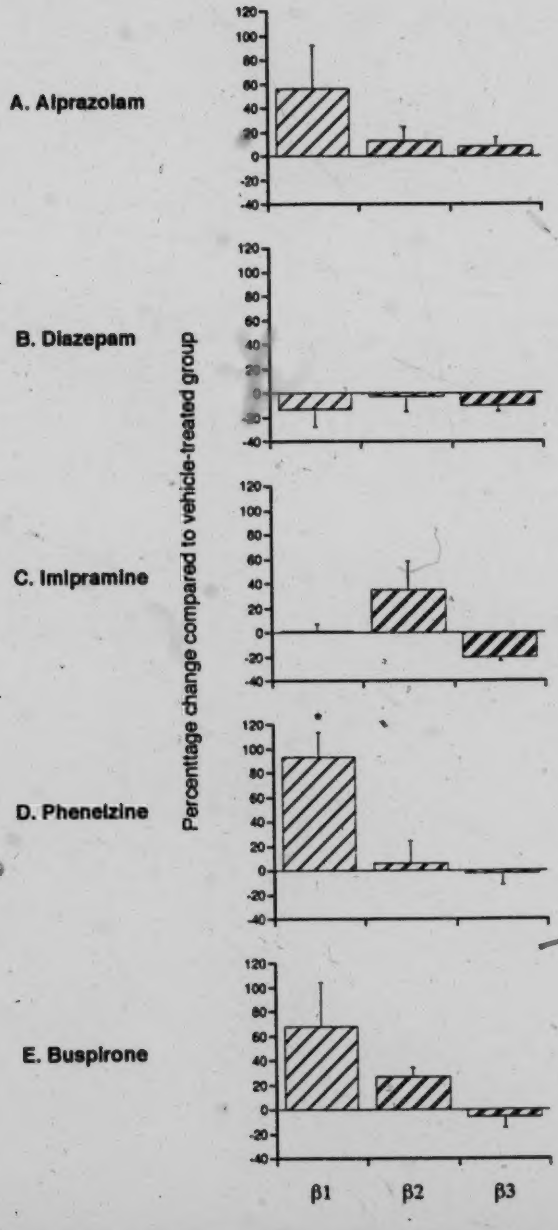

Figure 3.4 Effect of 21 days of drug treatment on cortex β-subunit gene expression Data represent the mean ± s.e.m. (n=6) percentage change in the various β-subunit mRNA levels relative to the vehicle-treated group. Statistical analyses were performed by the Dunnett's test pairwise comparison between drug- and vehicle-treated groups, * $P < 0.05$.

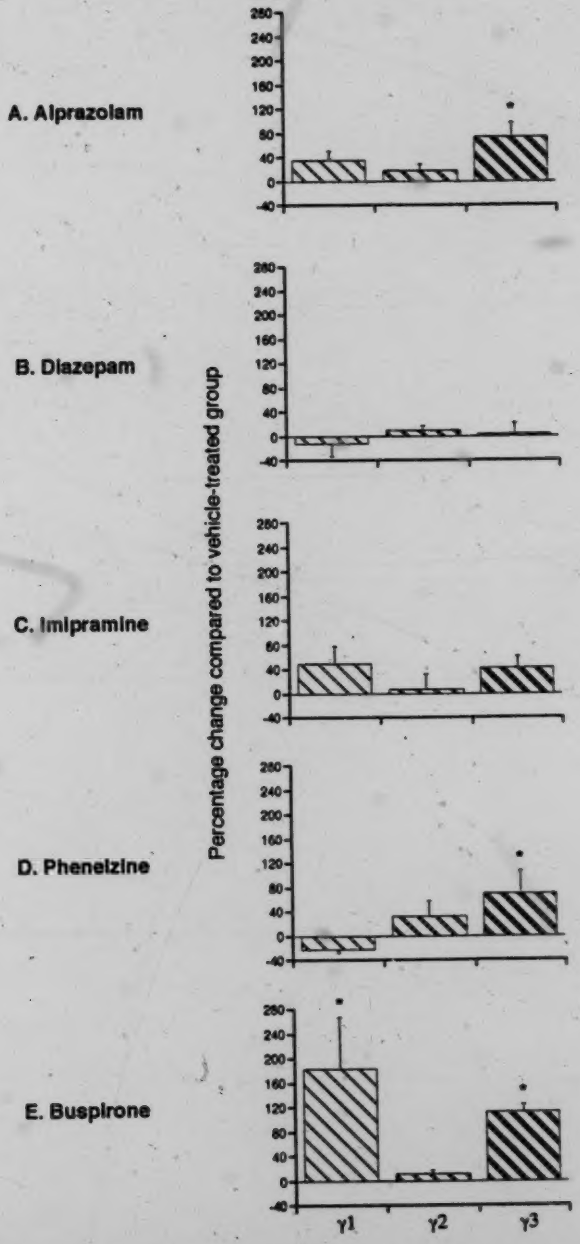

Figure 3.5 Effect of 21 days of drug treatment on cortex γ-subunit gene expression
Data represent the mean ± s.e.m. (n=6) percentage change in the various γ-subunit mRNA levels relative to the vehicle-treated group. Statistical analyses were performed by the Dunnett's test pairwise comparison between drug- and vehicle-treated groups, * $P < 0.05$.

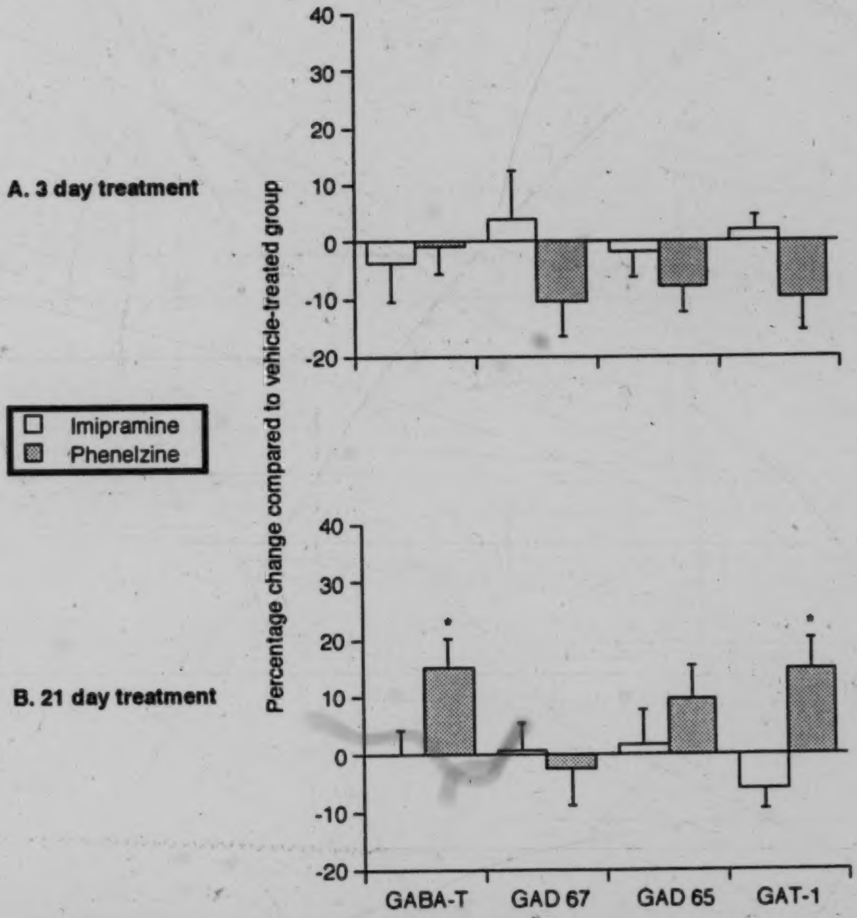

Figure 3.6 Effect of short- and long-term treatment with phenelzine or imipramine on GABA-T, GAD_{67}, GAD_{65} and GAT-1 mRNA levels
Data represent the mean ± s.e.m. (n=6) percentage change in GABA-T (GABA transaminase), GAD (glutamic acid decarboxylase) and GAT (GABA transporter-1) mRNA levels relative to the vehicle-treated group. For each time point, statistical analyses were performed with the Dunnett's test pairwise comparison between drug- and vehicle-treated groups, * $P < 0.05$.

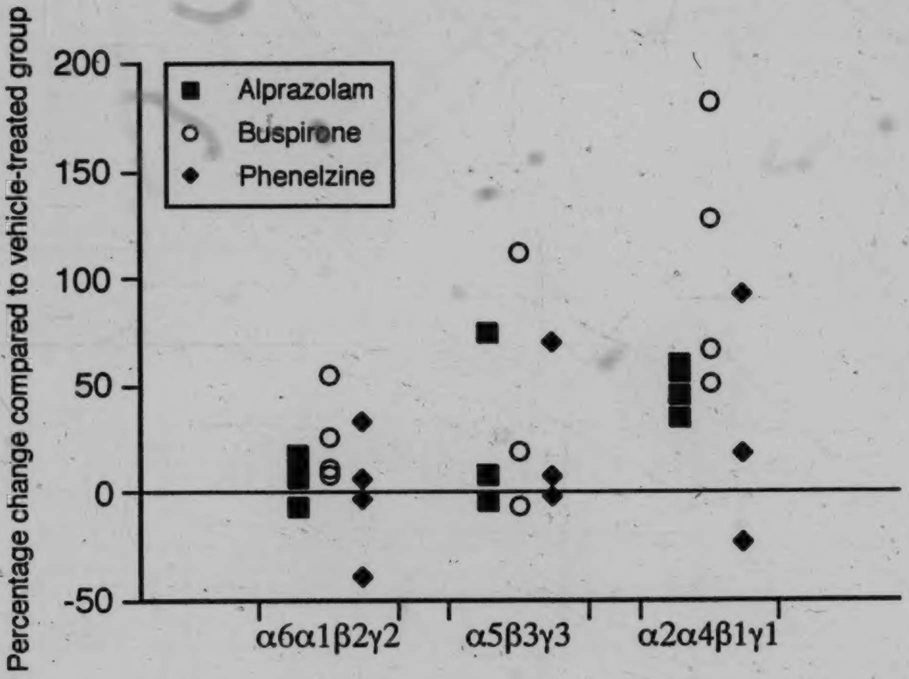

Figure 3.7 Effect of chronic drug treatment on GABA$_A$ receptor subunit gene cluster expression in cortex
For each drug that induced significant alterations in GABA$_A$ receptor subunit gene expression, the mean percentage changes in subunit mRNA level were grouped by gene cluster. The data represent the individual mean percentage in subunit mRNA levels that correspond to a particular gene cluster.

3.3. DISCUSSION

The validation experiments established that there was a linear relationship between the integrated band intensity and the amount of radioactivity present in the gel (Figure 3.1) and that the oligonucleotide probe concentration was sufficient to give a linear detection response over the range 0 - 20 μg of total cortex RNA (Figure 3.2). However, the relationship between exposure time and band intensity was not linear. Therefore, band intensities produced by different autoradiographic exposures of the same gel could not be compared. Hence, the specific activities of the different oligonucleotides used within one experiment were adjusted such that these would result in band intensities within the linear range (0 - 6,000 units) in a single autoradiographic exposure. Finally, 10 μg of total RNA of each sample were used for each experiment, and to ensure adequate quantification, integrated band optical intensities outside the range 20 - 5,000 were excluded.

With regard to the observed effects of the various chronic treatments on cortical $GABA_A$ receptor subunit steady-state mRNA levels, few significant effects were found. This may be due to the small number of animals per drug-treated group, as some changes fell just short of significance. Indeed, groups n numbers are used to calculate the variances of the means, the number of degrees of freedom used in the F-test to determine the mean square error and the critical value for statistical significance. Thus, small n numbers increase group variances, and by decreasing the number of degree of freedom, raise the mean square error and the significance critical values that result in a non-significant difference.

It is perhaps not surprising that alprazolam induced changes in the receptor subunit mRNA levels, since many laboratories have reported that chronic administration of $GABA_A$ receptor ligands produced changes in the steady-state mRNA levels of the receptor subunits (Baumgartner et al., 1994; Heninger et al., 1990; Herbison and Fenelon, 1995; Kang and Miller, 1991; Mhatre and Ticku, 1994b; Montpied et al., 1991; O'Donovan et al., 1992; Primus and Gallager, 1992; Zheng et al., 1996; Ticku and Mhatre, 1992; Tseng et al., 1994). In contrast, the finding that phenelzine and buspirone administrations also induced alterations in the receptor subunit gene expression is novel. The fact that diazepam, which was our positive control, did not induce significant changes in $GABA_A$ receptor subunit gene expression was unexpected. Data from other members of our research group suggest that diazepam induces changes in cortical $GABA_A$ receptor subunit mRNA levels at 7 days which peak at 14 days (Holt et al., 1996), and these changes are completely reversed by 28 days of treatment (Holt, 1998).

Thus, the changes observed in the present study are consistent with a waning of the effects of diazepam on $GABA_A$ receptor subunit gene expression toward a return to baseline values. Further, the magnitude of changes observed here for the cortical $\alpha1$- and $\beta1$-subunit gene mRNAs (9±8% and 14±14% decreases, respectively) is similar to the changes which have been reported elsewhere for the same subunits (24±4% and 3±15% decreases in $\alpha1$- and $\beta1$-subunit mRNA levels, respectively), after the same duration of treatment (Heninger et al., 1990). The main differences between this study and the work of Heninger et al., reside in the number of animals per treatment group (n=6 for our study, versus n=8-12 for the other published results) and in the statistical analysis. The data presented in this chapter were analyzed with Dunnett's pairwise comparison between treatment and control groups, while Heninger et al. (1990) performed repeated Student *t*-tests of treatment versus control groups. The differences in the number of animals per groups and in the statistical analysis may explain the fact that these authors obtained significant differences, whereas the changes (of similar sizes) observed in the present study did not reach the 5% level of significance. Again, a difference in the group n number not only change the variance of the mean, but also the mean square error in addition to the number of degree of freedom used for the determination of the statistical critical value. Thus, the changes in subunit gene expression described in this chapter may have been statistically significant, had the number of animal per group been of 10.

Overall, buspirone and alprazolam were the treatments which induced the biggest changes in $GABA_A$ receptor subunit gene expression, and seemed to produce a global increase in the amount of these subunit mRNAs. In contrast, imipramine and diazepam treatments did not seem to induce many alterations in the level of $GABA_A$ receptor subunits. Phenelzine treatment produced discrete large changes which are difficult to interpret and extrapolate into possible changes in the number and/or in the subunit composition of $GABA_A$ receptors. Thus, compared to buspirone, the antipanic drugs did not seem to produce any common effect on cortical $GABA_A$ receptor subunit mRNA levels. Finally, it is interesting to note that the two benzodiazepines displayed very different effects on β-subunit gene expression.

These data are the first of their kind to report such an effect of buspirone. The mechanism of action of buspirone, an anxiolytic, is thought to be mediated via a high affinity binding at presynaptic and postsynaptic serotonin $5\text{-}HT_{1A}$ receptors, and a lower affinity recognition of presynaptic dopamine D_2 receptors (Fulton and Brogden, 1997; Tunnicliff, 1991). Buspirone does not seem to interact directly at $GABA_A$ receptors, so buspirone effects on $GABA_A$ receptor subunit gene expression are likely to be consequential to a mechanism which remains to be determined.

The effects of imipramine and phenelzine on the steady-state mRNA levels of GABA-T, GAD_{65} and GAD_{67}, and GAT-1 were investigated to unveil any secondary mechanism triggered by the drug treatments which would further elucidate the effects of these drugs on GABAergic transmission. The data reported here are in general, in accordance with the data published previously (Lai et al., 1998). However, some minor differences can be observed between the two data sets in the average changes in GAD_{65} mRNA levels after imipramine treatments (values altered from 2% increase (Lai et al., 1998) to 2% decrease for the 3 day time point, and from 3% decrease (Lai et al., 1998) to 2% increase after 21 days of treatment). However, these small differences were not accompanied by alterations in the statistical significance of the variations in mRNA levels. More numerous were the differences in the mean changes in the various mRNA species amounts after phenelzine treatment. After 3 days of treatment, the average change in gene expression of GAD_{67} was altered from 3% increase to 10% decrease, that of GAD_{65} went from 2% increase to 8% decrease, and that of GAT-1 varied from 1% decrease to 10% decrease; while after 21 days of treatment, the mean change in gene expression of GAD_{67} was altered from 7% increase to 2% decrease, that of GAD_{65} went from 18% increase to 10% increase, and that of GAT-1 varied from 23% increase to 15% increase. Again, these differences were not accompanied by alterations in the statistical significance of the variations in mRNA amounts. Only one set of data reached the 5% level of statistical significance in this analysis compared to the previous one, that is the variation in GABA-T mRNA levels after chronic phenelzine treatment which changed from a average 20% increase (Lai et al., 1998) to a mean 15% increase in this analysis. This difference in statistical analysis result can be explained by the fact that the data presented here is the pooled result of separate experiments. Hence, the variation in GABA-T mRNA level after chronic phenelzine treatment changed from 20 ± 11% (Lai et al., 1998), to 15 ± 5%. Therefore, the pooling of the results from separate experiments caused a reduction in the variance which has probably contributed to the data reaching statistical significance. The decision to pool the data stemmed from the observation that for each treatment x parameter data, there was an inter-experiment variation ranging from 0.03 % to 32.57 % of the pooled treatment x parameter mean (median 4.39%, third quartile 14.88% for this data set). Thus, the experiments were replicated in order to obtain a more precise determination of the alterations in mRNA levels. Another factor that might have contributed to a differential statistical outcome is that the statistical analysis was previously performed by multiple Student *t*-test, whereas this analysis used Dunnett's pairwise comparison. As explained in Chapter 2, the choice of Dunnett's pairwise comparison was made in order to avoid the statistical errors inherent to multiple *t*-tests, and on the basis that Dunnett's test is designed to limit such errors.

Immunocytochemical studies in adult rat brain revealed that GAD_{65} is located at the nerve terminal, whereas GAD_{67} is prominently located in neuron cell bodies (Esclapez et al., 1994), suggesting that the two forms of GAD may carry out different functions. Since the rate-limiting step in the synthesis of GABA is the conversion of glutamate into GABA by the enzyme GAD, it was expected that the regulation of this enzymatic reaction, by means of changes in the expression levels of these enzymes, may be a target for the action of these drugs. To our surprise neither the duration of treatment nor the type of drug treatment influenced the expression of the GAD_{65} or GAD_{67}. Evidence suggests that about 50% of GAD exists as an apoenzyme (not bound to pyridoxal 5'-phosphate and not active), and that there is a cyclic interconversion between the apoenzyme and the holoenzyme (bound to pyridoxal 5'-phosphate and active) which controls the levels of active enzyme (Martin and Rimvall, 1993; Itoh and Uchimura, 1981). The fact that GAD operates at a small fraction of its maximal enzymatic activity and that there is a large fraction present as apoenzyme suggest that GAD is regulated on a short-term scale through GABA levels and its interaction with pyridoxal 5'-phosphate to respond to additional demand for GABA synthesis (Martin and Rimvall, 1993). Hence, there is a large excess of GAD protein present in the brain to meet the baseline demand for GABA synthesis. Further, long-term administration of γ-vinyl-GABA leads to a reduction in cortex GAD_{67} protein levels, without changes in the corresponding mRNA levels (Rimvall et al., 1993), suggesting that changes in the protein stability or some post-transcriptional mechanism(s) may be involved. Thus, there may be no need for GAD_{65} and GAD_{67} steady-state mRNA levels alteration to compensate for the treatment with phenelzine or imipramine. Therefore, the examination of the relative protein levels of GAD_{67} versus GAD_{65}, and apoenzyme versus holoenzyme, may be more appropriate to determine the effects of these treatments on GABA synthesis.

The results reported here show that chronic phenelzine treatment increased the steady-state mRNA levels of GABA-T. In our administration paradigm, GABA levels in the spinal cord of phenelzine-treated animals were 195% that of their controls. In a similar administration regime of phenelzine, GABA levels were 154 % that of their controls, and the dose-dependent inhibition of GABA-T plateaued at 25-35% (McManus et al., 1992). Further, even at high doses of phenelzine which increase GABA levels 3-4 times, the inhibition of rat brain GABA-T did not exceed 50% (Baker et al., 1991; McManus et al., 1992). In addition, a minimum of 2 mM of GABA is required to half saturate the enzyme (Fowler et al., 1983), suggesting that there is a large backup pool of GABA-T. Hence, the 15% increase in GABA-T we observed only after long-term administration may be sufficient to overcome the inhibitory effects of phenelzine on GABA-T and increase GABA turnover in treated animals in response to the elevated GABA concentration.

The location of the high-affinity sodium-dependent GAT-1 at the presynaptic nerve terminal or at the membrane of neighboring glial cells suggests that in addition to its role in the ending of synaptic GABA action by reuptake into the GABAergic terminals, GAT-1 influences both excitatory and inhibitory neighboring transmissions by modulating the paracrine effects of GABA, and that astrocytes may play an important role in this process (Minelli et al., 1995). Here again, acute or chronic treatment with imipramine did not alter the mRNA levels of GAT-1. These results detract from those expected from a drug and its metabolite that both increase GABA release (Korf and Venema, 1983). Further, imipramine does not inhibit any GAT subtypes (Nakashita et al., 1997), whereas desipramine, the primary metabolite of imipramine, inhibits all GAT subtypes at similar concentrations (Nakashita et al., 1997). These authors used 0.1 to 100 μM of these drugs (imipramine and desipramine) and showed that at concentrations around 1 μM the inhibition exerted is of 40% or less (Nakashita et al., 1997), whereas the drug levels reached in our paradigm ranged from 1.2 to 1.8 μg/g of spinal cord. These concentrations would correspond to 4-6 μM tissue concentration. Therefore the drug levels reached in the present paradigm relate to the low end of the spectrum of drug concentrations tested that produce a small inhibition of GAT-1 (Nakashita et al., 1997). A way to reconcile the present findings with the effects of imipramine and desipramine on GAT-1 function and GABA release is by suggesting that these two phenomena (i.e. inhibition of GABA reuptake and stimulation of GABA release) have opposite effects on GAT-1 gene expression, and/or that post-transcriptional mechanisms may be involved in the regulation of GAT-1 levels.

In contrast, chronic administration of phenelzine increased the mRNA levels of GAT-1 by 15%. Taking into account the distribution of GAT-1 on neurons and astroglia (Itouji et al., 1996; Minelli et al., 1995; Yasumi et al., 1997), this increase in GAT-1 gene expression may reflect an augmentation in GABA reuptake at the nerve terminal and/or in the glia cells. The fact that chronic but not acute phenelzine treatment induces such an effect suggests that this increase in GAT-1 gene expression may be part of a long-term mechanism enhancing GABA turnover and aimed at compensating for the long-lasting effects of phenelzine on brain GABA levels. Finally, the examination of the location of this rise in GAT-1 mRNA levels to either neuronal or glial cells may determine which of the synaptic or paracrine effects of GABA is regulated after chronic phenelzine treatment.

The mapping of the location of $GABA_A$ receptor subunit genes on the human genome has revealed that in general, these genes are grouped by $\alpha/\beta/\gamma$ clusters (Whiting et al., 1995). Indeed, $\alpha2$-, $\alpha4$-, $\beta1$- and $\gamma1$-subunit genes have been localized to the chromosome 4p13-4q11 (Buckle et al., 1989; Kirkness et al., 1991; Wilcox et al., 1992; McLean et al., 1995), $\alpha6$-, $\alpha1$-, $\beta2$ and $\gamma2$ subunit genes to chromosome 5q31-5q35 (Wilcox et al., 1992; Hicks et al., 1994; Russek and Farb, 1994) and $\alpha5$-, $\beta3$- and $\gamma3$-subunit genes to chromosome 15q11-15q13 region (Wagstaff et al., 1991b; Wagstaff et al., 1991a; Knoll et al., 1993; Greger et al., 1995). The $\alpha3$-subunit gene has been located on the human chromosome Xq28 region which also contains $\beta4$- and ε-subunit genes (Buckle et al., 1989; Wilke et al., 1997). Although no chromosomal map is available for the rat $GABA_A$ receptor subunit genes, the fact that the sequences and structures of these gene (Burt, 1994; Barnard et al., 1993; Whiting et al., 1995) and their organization (Lasham et al., 1991; Barnard et al., 1993) are highly preserved amongst vertebrates suggest the presence of homologous gene clusters in these species. Indeed, evidence indicates that in human and mouse, numerous gene loci are homologous (Searle et al., 1987), and that $GABA_A$ receptor subunit gene clusters are preserved (Danciger et al., 1993; Nakatsu et al., 1993; Wagstaff et al., 1991a)[*].

The fact that chronic treatment induced drug-specific patterns of alterations in $GABA_A$ receptor subunit gene expression, and that these genes are grouped by clusters led to the possibility that the discrete changes observed may be related to a coordinated effect on specific gene clusters. Indeed, findings from our group suggest that some drugs produce coordinated alterations in $GABA_A$ receptor subunit mRNA levels within discrete gene clusters (Holt et al., 1996). However, the experimental variation observed on the multiprobe solution hybridization assay, may lead to the situation where changes pertaining to a global effect on a specific gene cluster may not appear statistically significant for all the subunit genes included in that particular cluster. Therefore, the inclusion criteria for the analysis of the distribution of the alterations in $GABA_A$ receptor subunit mRNA levels grouped by gene cluster was that a particular drug treatment had to induce at least one significant change in subunit mRNA levels. The distribution of the percentage variation in $GABA_A$ receptor transcript levels grouped by gene cluster is presented in Figure 3.7.

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[*] **Danciger et al., (1993) described the location of an $\alpha4$-subunit which is now referred to as the $\alpha5$ subunit.**

The data revealed that the spread of the alterations induced by chronic phenelzine treatment was large and not indicative of any targeted effect on a specific gene cluster since the mean changes were distributed on each side of the 0% variation for the 3 gene clusters. In contrast, both alprazolam and buspirone displayed a smaller data scatter for the $\alpha6\alpha1\beta2\gamma2$ gene cluster, and a coordinated increasing effect on the expression of the $\alpha2\alpha4\beta1\gamma1$ gene cluster. The latter result regarding the effects of alprazolam on the $\alpha2\alpha4\beta1\gamma1$ gene cluster may somewhat be surprising since no significant effect were found for any of the subunits included in this gene cluster. However, this non statistical significance should be put into prospective with the fact that all changes in $\alpha2\alpha4\beta1\gamma1$ gene cluster mRNA levels induced were in the same direction and of similar amplitude. For comparison, phenelzine that induced a significant increase in $\beta1$-subunit mRNA levels, produced both positive and negative changes in the gene expression of the other members of the $\beta1$-subunit containing cluster. Hence, while the antidepressant, phenelzine, did not produce major common effects on $GABA_A$ receptor gene cluster expression, the anxiolytic drugs seemed to increase $GABA_A$ receptor subunit mRNA levels, especially those of the $\alpha2\alpha4\beta1\gamma1$ gene cluster. As a final remark, it should be noted that phenelzine which induced a significant increase in $\beta1$-subunit mRNA levels, has been recently shown to possess some anxiolytic properties (Paslawski et al., 1996). Therefore the effect of this drug on two out of the three measured $\alpha2\alpha4\beta1\gamma1$ gene cluster mRNA levels may be relevant to the anxiolytic profile of this drug.

The data presented here lead to the suggestion that an increase in the $\alpha2\alpha4\beta1\gamma1$ gene cluster expression may be related to the anxiolytic effect of these drugs. This theory may be supported by several facts. Diazepam, which possesses anxiolytic properties, also increases the expression of this gene cluster in the cortex after 7 and 14 days of treatment by 43 and 26% respectively (Holt et al., 1996). However, after 28 day of treatment, the level of expression of this gene cluster returned to control values (Holt, 1998). Zolpidem, another sedative-hypnotic drug, also increases $\alpha2\alpha4\beta1\gamma1$ gene cluster expression after 7 or 14 days of treatment (Holt et al., 1997), and as for diazepam administration, the maximal changes are observed after 7 days of drug delivery (Holt et al., 1996; Holt et al., 1997). Therefore, the results described in this chapter are consistent with those mentioned above that show a transient effect of diazepam on the $\alpha2\alpha4\beta1\gamma1$ gene cluster expression, reversed to baseline values by 28 days of treatment. Further, buspirone is an anxiolytic which requires chronic administration (2 weeks on average) for the establishment of its onset of action (Fulton and Brogden, 1997). The anxiolytic effects of this drug are sustained over the long-term for treatment periods lasting up to one year (Fulton and Brogden, 1997). Compared to the benzodiazepines, buspirone has a lower potential for inducing sedation, psychomotor and/or cognitive impairments (Fulton and Brogden, 1997). Finally, this drug has a low potential for dependence and abuse and does not produce cross-tolerance with the benzodiazepines or withdrawal symptoms (Fulton and Brogden, 1997). Recent evidence suggests that benzodiazepine tolerance may be related to alterations in specific $GABA_A$ receptor subunit gene expression (Holt et al., 1996; Holt et al., 1997; Holt, 1998). Hence, it can be speculated that the changes in $GABA_A$ receptor $\alpha2\alpha4\beta1\gamma1$ gene cluster expression observed upon chronic treatment with buspirone (which again does not present psychomotor and cognitive impairments, tolerance or a withdrawal side effect profile) may be linked to the anxiolytic effects of this drug.

The anxiolytic effects of drugs, such as diazepam, zolpidem and alprazolam, that interact with $GABA_A$ receptors occur very quickly. However, it is known that upon prolonged ligand exposure, receptors desensitize and are removed from the cell surface. Thus, the maintenance of the array of anxiolytic effects exerted by drugs that act at $GABA_A$ receptors may be mediated by the rapid induction of changes in the receptor subunit gene expression (i.e., as for diazepam after 7 or 14 days of treatment). In addition, tolerance could develop as a result of a return to baseline of the alterations induced, or by further distinct effects on gene expression (i.e., after 14 days of diazepam administration). As mentioned above, both diazepam and zolpidem increase rapidly and transiently the expression of the $\alpha2\alpha4\beta1\gamma1$ gene cluster (Holt et al., 1996; Holt et al., 1997; Holt, 1998). Hence, a change in the $\alpha2\alpha4\beta1\gamma1$ gene cluster expression may underlie a specific aspect of the anxiolytic effects produced by these drugs that is also shared with buspirone.

Consistent with such an hypothesis, the difference between the time of onset of action of anxiolytic agents acting at $GABA_A$ receptors, and buspirone seems to correlate with the delayed appearance of the changes in $\alpha2\alpha4\beta1\gamma1$ gene cluster expression after buspirone administration. Subsequent reversal of this increase in the $\alpha2\alpha4\beta1\gamma1$ gene cluster expression (as seems to be the case for diazepam and zolpidem) may be related to the development of tolerance to these anxiolytic effects. To further support this hypothesis, drugs that display behavioral anxiolytic properties shared with buspirone should increase $\alpha2\alpha4\beta1\gamma1$ subunit gene cluster mRNA levels in the cortex. In addition, over a long-term treatment, buspirone would maintain the increase in the $\alpha2\alpha4\beta1\gamma1$ gene cluster expression over an extended period of time, whereas the effect of drugs that produce tolerance would be transient. Again, correlation of behavioral tests investigating the development of tolerance with the molecular biology studies assessing the mRNA levels of the $\alpha2\alpha4\beta1\gamma1$ subunit gene cluster would strengthen this hypothesis.

Immunohistochemical studies revealed that the distribution of $GABA_A$ receptor subunit gene expression in the cortex is segregated between lamina (Fritschy et al., 1998). Indeed, $\alpha2$-subunit immunoreactivity is mostly detected in the axon initial segments of layer II-III pyramidal cells (Fritschy et al., 1998), which are thought to be involved in the processing of the somatosensory inputs to the cortex (Berman et al., 1992; Ebner and Armstrong-James, 1990). The axon initial segment of pyramidal cells, which is the region where action potentials are initiated, is innervated by GABAergic chandelier cells (Berman et al., 1992; Barnstable et al., 1992). Thus the chandelier neuron activity seems to be a potent regulator of the pyramidal cell firing rate (Berman et al., 1992; Barnstable et al., 1992). Consequently, it can be extrapolated that anxiolytic agents may alter the chandelier cell GABAergic gating of the processing of the peripheral inputs to the cortex.

To summarize, the changes in $GABA_A$ receptor subunit mRNA levels we observed in the cortex after 21 day treatment with antidepressant/antipanic and anxiolytic drugs did not reveal any changes specific to all the antipanic agents. In contrast, a change in the expression of the $\alpha2\alpha4\beta1\gamma1$ gene cluster in the cortex seemed specific for the anxiolytic drugs. In addition, although imipramine and phenelzine seem to increase GABA transmission, only chronic phenelzine administration triggered changes in the expression of GABA-T and GAT-1. Therefore, the lack of effect of imipramine on molecules involved in GABAergic transmission remains to be elucidated, but our results suggest that the long-term effects of phenelzine on GABAergic transmission may trigger compensatory mechanisms to enhance GABA turnover.

4. Effect of chronic antipanic drug treatment on GABA$_A$ receptor subunit gene expression in the cerebellum[*]

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[*] A portion of this chapter has been submitted: Tanay V.A.-M.I., Greenshaw A.J., Baker G.B., Bateson A.N. (1998) Common effect of antipanic drug on cerebellar GABA$_A$ receptor α6-subunit gene expression: a molecular study.

4.1. INTRODUCTION

The cerebellum has a high concentration of GABA$_A$ receptors and has recently been proposed as a model system for studying GABA$_A$ receptor diversity (Wisden et al., 1996). As schematized below in Figure 4.1, this brain region is characterized by a small number of cell types present within well-defined neuronal circuitry. In addition, these cells express a small subset of GABA$_A$ receptor subunits which are differentially expressed between the various cell types. This allows the investigation of the physiological roles of GABA$_A$ receptor subtypes on different neurons and the correlation of the receptor subunit composition with the functional and pharmacological profile of the receptors.

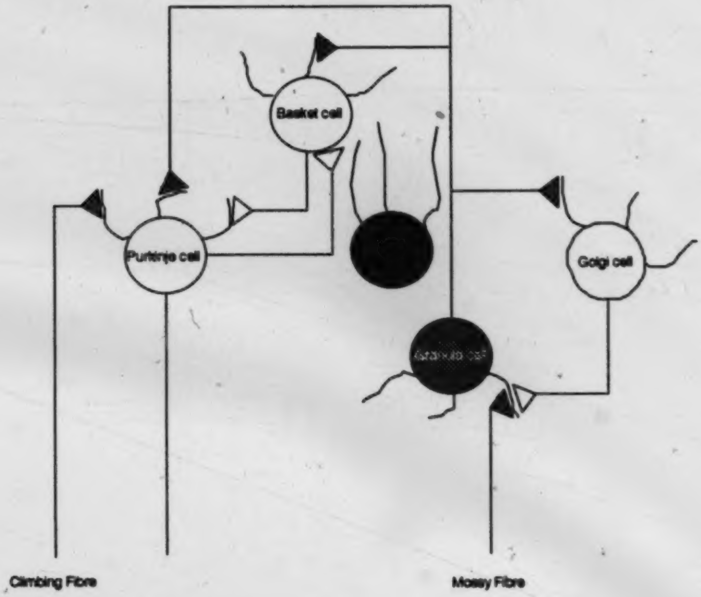

Figure 4.1 Schematic diagram of the main cerebellar cell types involved in the cerebellar pathways
Excitatory cells and terminals are represented as shaded circles and triangles, respectively. Inhibitory (GABA releasing) cells and terminals are depicted as open circles and triangles, respectively. Schematic adapted from (Ottersen, 1993) and (Wisden et al., 1996).

The cerebellar cortex consists of two layers: the molecular and the granule cell layer. The vertebrate molecular layer contains basket cells, Bergmann glial processes, Purkinje and Golgi cell dendrites and the axon terminals of the granule cells synapsing onto Purkinje, basket and Golgi cells (Ottersen, 1993; Wisden et al., 1996). The interface between the molecular and granule cell layer is composed of the Purkinje, Bergmann and Golgi cell bodies (Ottersen, 1993; Wisden et al., 1996). Excitatory glutamatergic inputs to the cerebellum are carried by the mossy or climbing fibers which origin mainly from the inferior olive and the pontine nuclei (Brodal and Bjaalie, 1992; Ottersen, 1993). The mossy fibers synapse onto granule cells which in turn activate 3 types of inhibitory cells: basket, Purkinje and Golgi cells (Ottersen, 1993; Wisden et al., 1996), whereas the climbing fibers activate the Purkinje cells. Cerebellar outputs are effected by the release of inhibitory neurotransmitter from the axon terminals of Purkinje cells (Ottersen, 1993; Wisden et al., 1996). The tone of this inhibition is determined by two separate reciprocal synapses. The first loop involves a negative feedback from Golgi to granule cells which regulates the granule cell output to the basket and Purkinje cell, and the second synaptic loop includes reciprocal inhibition between the basket and Purkinje cells. As stated above, these various cell types express different GABA$_A$ receptor subunit combinations. The $\alpha 1\beta 2\beta 3\gamma 2$ subunit combination is very abundant in the molecular-granule cell layer border and is mainly carried by the Purkinje cells (Whiting et al., 1995; Wisden et al., 1996). Basket cells express and $\alpha 1\beta 2\gamma 2$ whereas Bergmann glia express at low levels an $\alpha 2\gamma 1$ subunit combination (Whiting et al., 1995; Wisden et al., 1996). In contrast to the granule cells which strongly express $\alpha 1\alpha 6\beta 2\beta 3\gamma 2\delta$ subunits (Nusser et al., 1998; Jechlinger et al., 1998), Golgi cells are believed to contain an $\alpha 2\alpha 3\gamma 1$ subunit combination (Whiting et al., 1995; Wisden et al., 1996).

The two main pathophysiological theories of PD are the hyperactivity of the locus coeruleus and the abnormality of the respiratory centers, which both indicate a role for a dysregulation of brainstem nuclei (Papp et al., 1993; Charney et al., 1990b) but do not include the cerebellum. This omission probably stems from the fact that the cerebellum has been traditionally considered solely as a motor control center. However, there are data to support a role for the cerebellum in cognitive and language functions (Leiner et al., 1993), as well as psychiatric and mood disorders such as PD. Indeed, schizophrenia has been related to the impairment of the frontal-thalamic-cerebellar circuit (Andreasen et al., 1996; 1997) and in the mean rate of change in the volume of the right cerebellum (DeLisi et al., 1997). With regard to mood disorders, bipolar disorders have been associated with a cerebellar circuitry pathology (Soares and Mann, 1997), and depressed patients have been reported to display a decreased cerebellar size compared to healthy volunteers (Shah et al., 1992; Tien and Ashdown, 1992; Soares and Mann, 1997). Recent imaging studies in post-traumatic stress disorder patients also showed abnormal enhanced cerebellar activation during anxiety attacks (Brannan et al., 1997; Bremner et al., 1997).

PET studies, investigating brain activation foci by the determination of changes in cerebral blood flow, established that lactate-induced panic attacks in PD patient (versus controls) and CCK$_4$-induced anxiety in healthy volunteers produced activation of the cerebellar vermis (Reiman et al., 1989; Benkelfat et al., 1995). The authors of these studies did not discuss a possible role for cerebellum in fear-anxiety. Although one study investigated the possibility that the cerebellar activation might be due to adjacent brain structures (Reiman et al., 1989), and the other acknowledged some evidence for cerebellar involvement in fear behaviors in animals (Benkelfat et al., 1995) there was no mention that the cerebellum could be involved in a neuroanatomical model of fear-anxiety. The results from imaging studies investigating benzodiazepine receptor density with ^{123}I-iomazenil reported no alteration in the cerebellum of PD patients compared to matched healthy volunteers (Kuikka et al., 1995). These results may be explained by the following. ^{123}I-iomazenil is a benzodiazepine site ligand that is a structural analog of flumazenil (Ro 15-1788) and which binds to virtually all $\alpha\beta\gamma2$-like GABA$_A$ receptor subtypes (Johnson et al., 1990; Wisden et al., 1996). Therefore the measurement of ^{123}I-iomazenil binding is an assessment of the variation in the total number of benzodiazepine site-containing receptors but not of the variation in the proportion of specific receptor subtypes. Hence if PD patients display a variation in the proportion of a specific benzodiazepine receptor subtype, or in the amount of a benzodiazepine-insensitive receptor subtype, compared to the healthy controls, ^{123}I-iomazenil binding will show no difference between these patients and their controls.

As mentioned above, some psychiatric disorders, and anxiety in particular, may involve the cerebellum as part of a multi-brain regional pathway. Further, neuroanatomical data indicate that the cerebellar inhibitory output is the result of a poised interaction between excitatory and inhibitory loops. The cerebellum possesses numerous and various interconnections with brain regions and nuclei such as the cortex, limbic system and brainstem implicated in anxiety (Olson and Fuxe, 1971; Mason and Fibiger, 1979; Dietrichs and Haines, 1989; Dietrichs et al., 1994; Gorman et al., 1989). Indeed, the pontine nuclei output to the cerebellum constitutes the majority of the mossy fibers synapsing on the cerebellar granule cells (Brodal and Bjaalie, 1992). Evidence shows that the cerebellar cortex receives bi-synaptic projections from the corticopontine fibers projecting to the pontocerebellar pathway (Brodal and Bjaalie, 1992). In addition, some multi-layered afferents to the cerebellar cortex contain monoamines which are thought to modulate the excitatory and inhibitory cerebellar circuitry (Ottersen, 1993). Thus, the cerebellum may be a center of interest for the study of anxiety. Although controversy remains on a possible abnormality of cerebellar GABA$_A$ receptors in PD patients, it is possible that the therapeutic effect of antipanic agents is mediated by a modulation of the cerebellar inhibitory output to the brain areas thought to be involved in anxiety and panic. Since GABA$_A$ receptor-mediated transmission is the main cerebellar inhibitory pathway, I investigated the effect of chronic treatment with antipanic drugs on GABA$_A$ receptor subunit gene expression.

4.2. RESULTS

Changes in GABA$_A$ receptor subunit mRNA levels resulting from the 21 day treatment with the different drugs, expressed as mean±s.e.m. percentage change compared to the vehicle-treated group are shown in Figures 4.2 to 4.4. Although the RNA samples were tested for the presence of α5-subunit specific mRNA, no signal was obtained with the α5-subunit probe, consistent with the fact that α5-subunit expression is restricted to only a few brain areas, which do not include the cerebellum (Laurie et al., 1992; Wisden et al., 1992). The pattern of changes in α-subunit gene expression was drug specific (Figure 4.2). Compared to the vehicle-treated group, the number of significant changes revealed by Dunnett's test is restricted to a few α-subunit mRNAs. Alprazolam treatment significantly increased the levels of α6-subunit mRNA (37%), while imipramine administration significantly elevated the levels of α1-subunit (36%), α3-subunit (40%), and α6-subunit (30%) mRNAs (Figure 4.2). Figure 4.2 also reveals that, compared to buspirone, all antipanic agents tested increase α6-subunit gene expression. Phenelzine produced an increase (23%) in α6-subunit mRNA levels which just failed to reach significance, whereas buspirone altered α6-subunit mRNA levels by only 3%.

The changes in β-subunit gene expression levels are displayed in Figure 4.3. Only imipramine treatment showed any significant increase in β2-subunit mRNA levels (38%). Compared to buspirone, the antipanic agents did not commonly alter β-subunit gene expression.

Likewise, the changes in γ-subunit mRNA levels shown in Figure 4.4 reveal no significant effects except that of alprazolam on γ2-subunit gene expression (48%). It is clear from the examination of Figure 4.4 that no common effect of the antipanic agents arose, despite the fact that the two antidepressants commonly increased γ3-subunit gene expression (39% for imipramine, 33% for phenelzine) while the anxiolytics seem devoid of such effect on this subunit (2% change for alprazolam, and 7% decrease for buspirone).

The changes in GABA$_A$ receptor subunit gene expression grouped by gene cluster for a particular drug treatment are presented in Figure 4.5. The two antidepressants imipramine and phenelzine increased the gene expression of all the members of the α6α1β2γ2 and α5β3γ3 subunit gene clusters. Imipramine treatment also negatively regulated the expression of the α2α4β1γ1 subunit gene cluster. Finally alprazolam administration did not induced any striking coordinated effect on any of the gene clusters investigated.

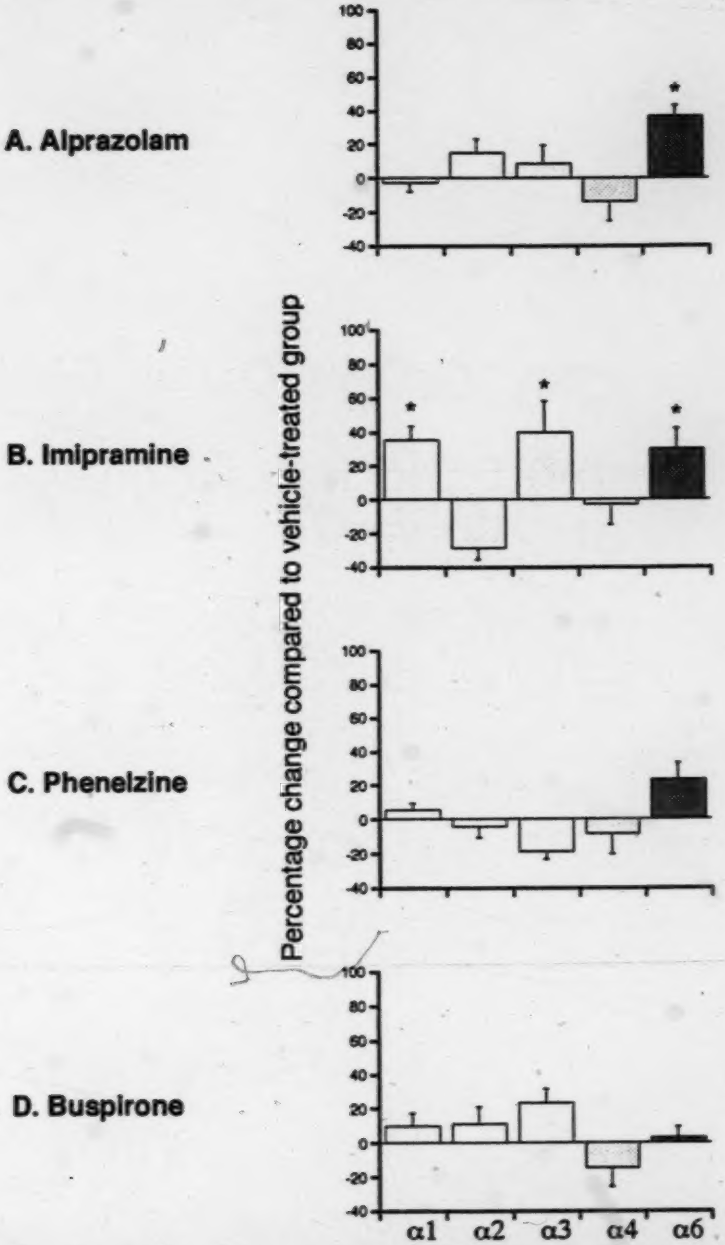

Figure 4.2 Effect of 21 day drug treatment on cerebellum α-subunit gene expression Data represent the mean ± s.e.m. (n=6) percentage change in the various α-subunit mRNA levels relative to the vehicle-treated group. Statistical analyses were performed by the Dunnett's test pairwise comparison between drug- and vehicle-treated groups, * $P < 0.05$.

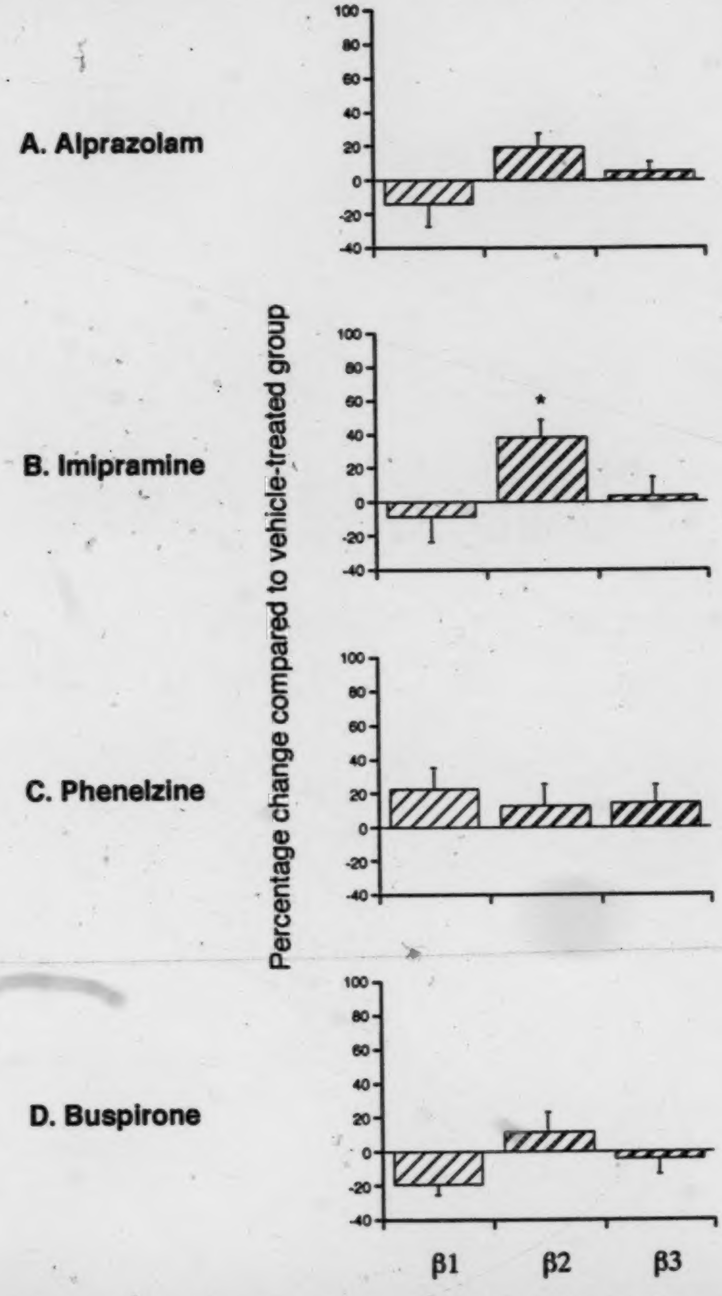

Figure 4.3 Effect of 21 day drug treatment on cerebellum β-subunit gene expression Data represent the mean ± s.e.m. (n=6) percentage change in the various β-subunit mRNA levels relative to the vehicle-treated group. Statistical analyses were performed by the Dunnett's test pairwise comparison between drug- and vehicle-treated groups, * $P < 0.05$.

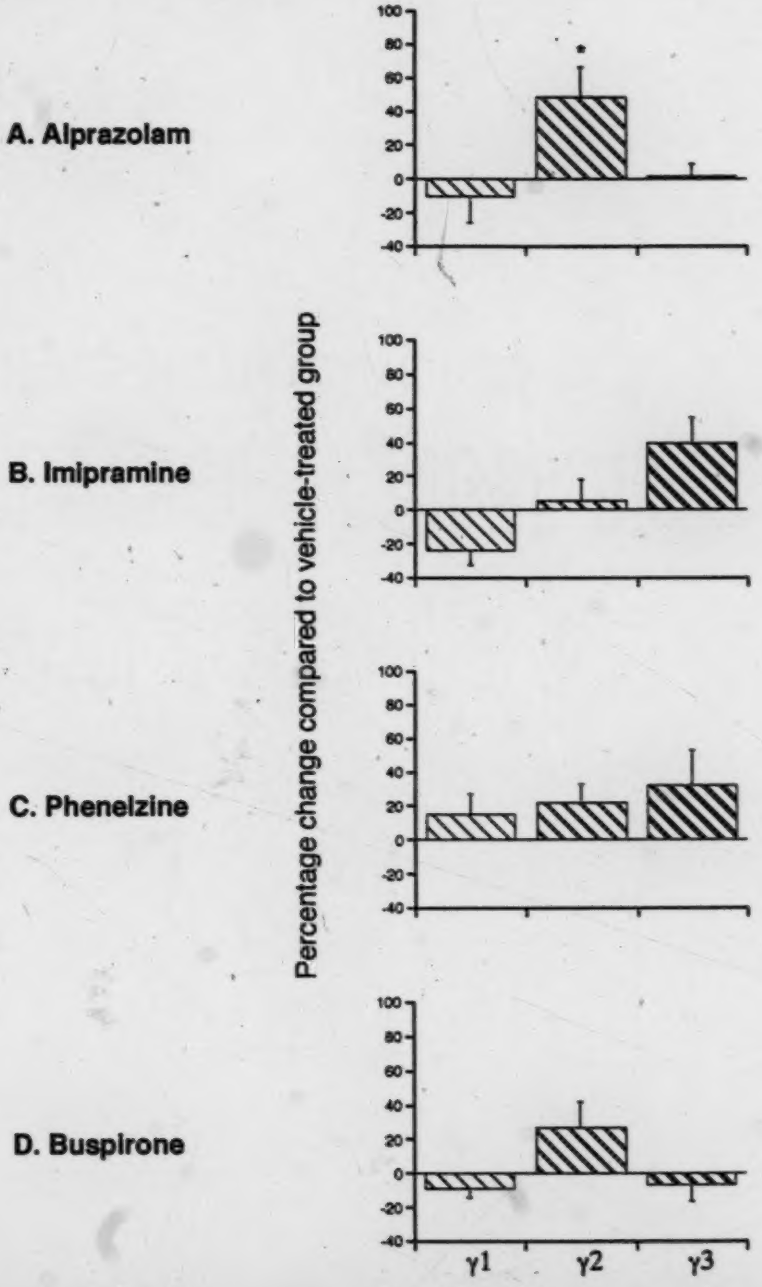

Figure 4.4 Effect of 21 day drug treatment on cerebellum γ-subunit gene expression
Data represent the mean ± s.e.m. (n=6) percentage change in the various γ-subunit mRNA levels relative to the vehicle-treated group. Statistical analyses were performed by the Dunnett's test pairwise comparison between drug- and vehicle-treated groups, * $P < 0.05$.

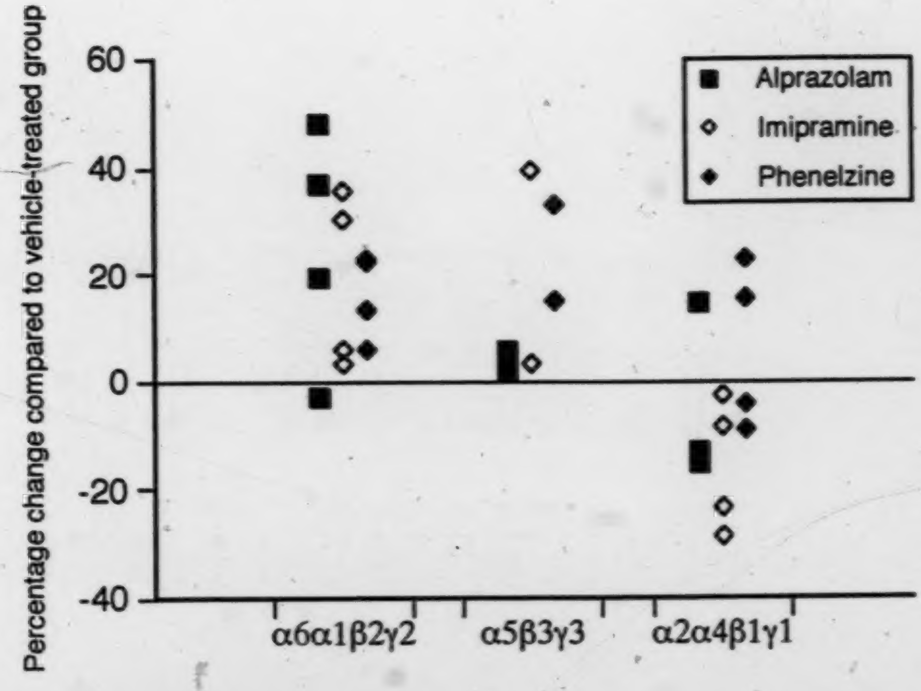

Figure 4.5 Effect of chronic drug treatment on GABA$_A$ receptor subunit gene cluster expression in cerebellum

For each drug that induced significant alterations in GABA$_A$ receptor subunit gene expression, the mean percentage changes in subunit mRNA level were grouped by gene cluster. The data represent the individual mean percentage in subunit mRNA levels that correspond to a particular gene cluster.

4.3. DISCUSSION

The results presented here are the first to describe the effect of chronic treatment with antidepressant/antipanic and anxiolytic agents in the rat cerebellum. Chronic treatment with these agents produced specific changes in the expression of GABA$_A$ receptor subunit genes in the cerebellum which are different from those observed in the cortex. In contrast to the cortex, where imipramine did not produce any effects on GABA$_A$ receptor subunit steady-state mRNA levels, this drug induced the largest number of significant changes in the cerebellum. Buspirone, which triggered many changes in cortical GABA$_A$ receptor subunit gene expression, did not produce any significant changes in the cerebellum. These findings argue against a role for the cerebellar GABA$_A$ receptors in generalized anxiety, but do not exclude the possibility of an involvement in fear-anxiety.

The determination of the effects of these drugs on the GABA$_A$ receptor subunit genes grouped by gene clusters is shown in Figure 4.5. Similar to the cortex, this representation of the data aims at pinpointing any coordinated effect of any drug on a specific gene cluster. The two antidepressants imipramine and phenelzine produced coordinated increases on the $\alpha6\alpha1\beta2\gamma2$ and $\alpha5\beta3\gamma3$ subunit gene clusters. In addition, imipramine produced a coordinated decreasing effect on the expression of the members of the $\alpha2\alpha4\beta1\gamma1$ gene cluster. Surprisingly, the common effect of antipanic drugs on $\alpha6$-subunit gene expression did not translate into a specific $\alpha6$-subunit containing gene cluster locus activation.

The observation that alprazolam, imipramine and phenelzine alter $\alpha6$-subunit mRNA levels in a similar manner compared to buspirone, lends support to the hypothesis of common changes in GABA$_A$ receptor subunit gene expression upon chronic treatment with antipanic agents. However, this common effect of these antipanic agents did not correspond to an upregulation in the expression of a specific gene cluster. Therefore, these results suggest that the increase in $\alpha6$-subunit gene expression induced by chronic treatment with antipanic agents might relate to the discrete upregulation of $\alpha6$-subunit containing receptors in the cerebellum.

As reviewed by Wisden et al. (1996) in adult naive rats, the $\alpha6$-subunit gene expression is restricted to the granule cell layer of the cerebellum. Cerebellum granule cells are also known to possess high levels of $\alpha1$-, $\beta2$-, $\beta3$-, $\gamma2$- and δ-subunits, and lower levels of $\alpha4$-, $\beta1$- and $\gamma3$-subunits (Laurie et al., 1992; Lüddens et al., 1990; Shivers et al., 1989; Gao and Fritschy, 1995). According to Khan et al. (1996), the $\alpha6$ subunit can coexist with the $\alpha1$ subunit in the same cerebellar GABA$_A$ receptor, although this concept had been excluded by others (Quirk et al., 1994b). However recent immunoprecipitation studies have demonstrated that 53% of the α6-subunit containing receptors also present an α1 subunit (Jechlinger et al., 1998). Therefore, the major GABA$_A$ receptor subtypes which are thought to be present in granule cells are: α1β2/3γ2, α6β2/3γ2, α6α1β2/3γ2, α6α1β2/3δ and α6β2/3δ (Wisden et al., 1996). Although it is well established that α6β2/3γ2 receptor subtypes are approximately 10 times more sensitive to GABA than the receptor composed of an α1β2/3γ2 subunit combination, controversy remains over the functional properties of α6α1β2/3γ2 receptor subtypes (Kleingoor et al., 1993; Mathews et al., 1994; Ducic et al., 1995; Saxena and Macdonald, 1996; Wisden et al., 1996). The α6α1β2/3γ2 receptor subtypes have been claimed to retain both types of benzodiazepine pharmacological properties (high affinity diazepam-sensitive and diazepam-insensitive binding of Ro 15-4513) associated with each subunit (Khan et al., 1996; Wisden et al., 1996), but mature granule cells in culture display functional properties virtually identical to those of the α6β2γ2 receptor subtype, and seem to be devoid of any functional expression associated with the α1-subunit (Mathews et al., 1994). Thus, it can be assumed that there would be co-dominance of α1- and α6-subunits for binding, but the α6-subunit would be the major functional determinant of α6α1β2/3γ2 receptor subtypes.

Assuming that the increase in α6-subunit mRNA levels is occurring in granule cells, the final result would be an increase in the number of receptors displaying α6β2/3γ2 receptor-like functional properties, such as a greater affinity for GABA and sensitivity to the inhibition by Zn^{2+}, than their α1-subunit containing counterparts (Ducic et al., 1995; Saxena and Macdonald, 1996). Such a change in receptor subtype may be accounted for by one or more of the following events: (1) a switch of the α1β2/3γ2 or α6α1β2/3γ2 receptor subtype to α6α1β2/3γ2 and/or α6β2/3γ2 receptor subunit composition, respectively, accompanied by an unaltered total number of GABA$_A$ receptors; (2) an increase in the number of α6β2/3γ2 and/or α6β2/3δ receptor subtypes while keeping the number of α1- and α6α1-subunit containing receptor subtypes constant, leading to an increase in the total number of GABA$_A$ receptors present. According to the first hypothesis, a mean increase of 30% in α6-subunit mRNA level would be compensated by an average 60% decrease in α1-subunit mRNA levels, since there is about twice as much α6-subunit mRNA in the cerebellum (this 2:1 α6:α1 subunit mRNA ratio stems from the fact that, in order to obtain the same band intensity on the autoradiograms, the specific activity of the α6-subunit specific antisense oligonucleotide has to be half of that of the α1-subunit antisense oligonucleotide). Regarding the second hypothesis, a mean increase of 30% in α6-subunit mRNA levels would be accompanied by a similar increase in β2-

and β3-subunit mRNA levels, taking into account that these subunits are also expressed in Purkinje cells and that there is twice as much β3-subunit mRNA (similarly to the α6:α1 mRNA ratio, the specific activity of the β3-subunit specific antisense oligonucleotide has to be half of that of the β2-subunit antisense oligonucleotide). The fact that α1-subunit mRNA level changes are far from the expected 60%, and that the addition of the changes in mRNA levels of β2- with β3-subunit approximate the level of changes in α6-subunit gene expression, favors the second hypothesis. However, compared to buspirone, the antipanic agents did not produce a common effect on γ2-subunit gene expression (Figure 4.3), suggesting that the α6β2/3γ2 GABA$_A$ receptor subtype is unlikely to be the shared target for these drugs. Therefore, it is possible that the increase in α6-subunit mRNA levels induced by the antipanic agents corresponds to an increase in the number of α6β2/3δ receptor subtype. Since the relative change in δ-subunit mRNA levels has not been determined, this hypothesis cannot be further considered in light of the present data.

The granule cells receive GABAergic input from Golgi interneurons and from the cerebellar nuclei, and excitatory glutamatergic input from the mossy fibers which originate from many brain areas (Wisden et al., 1996). A recent study showed that the synaptic junctions between cerebellar GABAergic Golgi cell and granule cells were immunoreactive for α1 and α6 subunits, whereas the synapses between cerebellar glutamatergic mossy fibers and granule cells contained only the α6 subunit (Nusser et al., 1996; Nusser and Somogyi, 1997). Hence, in cerebellar granule cells, the α6-subunit is present at both inhibitory and excitatory synapses, indicating that the α6 subunit may fulfill various functions. As rationalized above, the results obtained so far lead to the speculation that the observed increase in α6-subunit might reflect an augmentation in the number of α6β2/3δ GABA$_A$ receptor subtype. Since the distribution of δ-subunit is also restricted to the granule cell layer in the cerebellum (Benke et al., 1991; Persohn et al., 1992; Laurie et al., 1992; Fritschy and Mohler, 1995; Gao and Fritschy, 1995), chronic treatment with the antipanic drugs is hypothesized to elevate the levels of δ-subunit transcript by about 30%. Such a finding would be of special interest in the light of recent studies showing that the distribution of cerebellar GABA$_A$ receptor subtypes is segregated, so that α6β2/3δ GABA$_A$ receptor subtype appears to be present exclusively on extrasynaptic membranes of cerebellar granule cells (Nusser et al., 1996; Nusser et al., 1998). Functional studies have shown that α6β2/3δ GABA$_A$ receptor subtype displays a high affinity for GABA (Saxena and Macdonald, 1996), that δ-subunit containing receptors do not desensitize upon an extended exposure to an agonist (Saxena and

Macdonald, 1994), and that $GABA_A$ receptors of granule cells could mediate both phasic and tonic responses (Wall and Usowicz, 1997; Brickley et al., 1996). Hence, the phasic inhibition may be mediated by synaptic $GABA_A$ receptors, while the tonic inhibition results from the activation of extrasynaptic receptors (Wall and Usowicz, 1997; Brickley et al., 1996), such as the $\alpha6\beta2/3\delta$ receptor subtypes (Nusser et al., 1998). Therefore, the increase in $\alpha6$-subunit gene expression induced by the antipanic drugs may be related to an enhanced GABAergic tonic inhibition mediated by an increase in the number of non-synaptic $\alpha6\beta2/3\delta$ $GABA_A$ receptor subtype present on cerebellar granule cells.

The determination of the cellular localization of the occurrence of the increase in $\alpha6$-subunit mRNA levels may provide an insight into the $GABA_A$ receptor-mediated neurotransmission targeted by antipanic agents. Further studies are warranted to confirm that other antipanic agents (such as the SSRI, sertraline) also produce the same effect and that these changes in mRNA levels are translated into changes in protein levels. In addition to the measurement of the amount of $\alpha6$-subunit protein present after treatment, a comparison of the number of diazepam-insensitive binding sites with that of muscimol binding sites will determine which $\alpha6$-subunit containing receptor population is affected.

To summarize, the findings suggesting that antipanic drug common effects may involve the modulation of cerebellar $GABA_A$ receptor $\alpha6$-subunit gene expression contrasts with the proposed pathophysiological theories of PD. Although these results may not be directly linked to the pathophysiology or the pharmacotherapy of PD, they may provide a molecular basis for the investigation of such neurobiological processes. Hence alteration in cerebellar $GABA_A$ receptor $\alpha6$-subunit gene expression could be related to PD in three ways: (1) PD is linked to a decrease in $\alpha6$-subunit containing receptors in the cerebellum and antipanic agents increase this level back to normal, or (2) the primary mechanism of panic involves another neurotransmitter system, and the increase in the number of $\alpha6$-subunit containing receptors may counteract the effects of the abnormality in that neurotransmitter system, or (3) this increase in $\alpha6$-subunit gene expression may be shared by all antipanic agents but may not be involved in the therapeutic action, instead this effect may serve as a screening marker during the development of potential antipanic drugs. Further confirmation that this change in $\alpha6$-subunit mRNA level in the cerebellum is specific to antipanic agents, and corresponds to an increase in $\alpha6\beta2/3\delta$ receptor subtypes, may lead to the establishment of a more comprehensive model of the etiology/pharmacotherapy of PD, or alternatively validate the change in $\alpha6$-subunit mRNA levels as a molecular marker for antipanic efficacy.

5. Effect of chronic antipanic drug treatment on GABA$_A$ receptor subunit gene expression in the brainstem [*]

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[*] A portion of this chapter has been published: (Tanay et al., 1996).

5.1. INTRODUCTION

The phenomenology of PD has been broken into three consecutive phases: acute panic attack, anticipatory anxiety and phobic avoidance (Gorman et al., 1989). According to this model, it is the repetition of acute panic attacks which leads to the development of anticipatory anxiety of having another attack. In an attempt to alleviate the occurrence of panic attacks, the patients will develop a phobic avoidance towards situations where help is not readily available (Gorman et al., 1989). Acute panic attack, anticipatory anxiety and phobic avoidance are thought to originate from the activity of three distinct neuroanatomical regions: the brainstem, limbic lobe and prefrontal cortex respectively (Gorman et al., 1989).

The brainstem has been repeatedly implicated in the pathophysiological models of PD. Indeed, the range of somatic symptoms occurring during laboratory-induced and naturally-occurring panic attacks argue for hyperactivity of the autonomic nervous system. Although various panicogenic agents are used in laboratory settings, they all are thought to act via the brainstem, albeit at different levels. The main brainstem nuclei which have been involved are the nucleus tractus solitarius, the locus coeruleus and the periaqueductal gray. These nuclei have interconnections between them and with the medullary cardiovascular and respiratory centers (Gorman et al., 1989). Thus, activation of any of these nuclei may be responsible for the somatic symptoms associated with panic attacks.

Clinical studies looking at brainstem evoked potential and vestibular function in PD patients (Jacob et al., 1996; Knott et al., 1994) further supported a role for brainstem nuclei in PD. However, imaging studies aimed at the determination of the brain loci activated upon infusions with panicogenic agents such as lactate and CCK failed to report abnormal brainstem activation in PD patients (Benkelfat et al., 1995; Reiman et al., 1989). These results may be explained by the relative size of the brainstem nuclei compared to the resolution of the imaging technique used (PET). In addition, since many of the key brainstem structures are interconnected and receive input and/or project to cardiovascular and respiratory regulatory nuclei, the determination of the specific involvement of one or several nuclei by imaging techniques may be impossible with the technology currently available.

Evidence for the implication of GABA in PD has been reviewed in Chapter 1. GABA is a ubiquitous neurotransmitter in brainstem nuclei, especially in those thought to be of primary importance for anxiety and panic-like reactions. Thus, GABAergic transmission is of special interest regarding the etiology and/or the pharmacotherapy of PD. However, plasma and CSF GABA levels of PD patients before and after antipanic treatment are not different from healthy volunteers (Rimón et al., 1995; Roy-Byrne et al., 1992; Goddard et al., 1996). Although evidence supports a decreased benzodiazepine receptor function in anxious patients (Roy-Byrne et al., 1989; Roy-Byrne et al., 1990; Roy-Byrne et al., 1996; Cowley et al., 1995; Malizia et al., 1995), studies investigating benzodiazepine binding in PD patients concentrated on cortical areas and did not examine the receptor density in the brainstem (Kuikka et al., 1995; Schlegel et al., 1994; Kaschka et al., 1995; Malizia et al., 1997). This may be due to the small number of cell bodies and dendrites scattered within the white matter (Fritschy and Mohler, 1995), which is thought to limit the resolution of PET scans in the brainstem, rendering it more difficult to detect the investigated parameter (Reiman et al., 1989). However, brainstem GABA$_A$ receptors may be one of the key elements in the etiology and/or pharmacotherapy of PD. The brainstem is anatomically defined as including the midbrain, pons and medulla regions. The majority of the nuclei involved either putatively in PD or in the regulation of the cardiovascular and respiratory functions are located in the pons-medulla area. Hence, alteration of GABA$_A$ receptor subunit gene expression after 21 days treatment with antipanic drugs was investigated in the pons-medulla region.

5.2. RESULTS

The effects of the chronic drug treatment with various antipanic drugs on α-subunit mRNA levels are presented in Figure 5.1. Many significant changes were determined, but most of them pertained to the imipramine-treated samples. Alprazolam significantly increased the level of expression of the α2-subunit gene (76%), whereas imipramine significantly augmented α1-, α4-, α5- and α6-subunit mRNA levels by 57, 80, 108 and 238% respectively. In contrast, buspirone significantly decreased α1-subunit steady-state mRNA levels by 47%. It is interesting to note that imipramine and alprazolam produced either increases or no changes, whereas buspirone produced decreases or no change in α-subunit gene expression. Phenelzine had no significant effects on GABA$_A$ receptor α-subunit gene expression.

The variation in β-subunit gene expression after chronic administration of the different drugs is shown in Figure 5.2. Alprazolam treatment significantly decreased the levels of β2-subunit mRNA by 24%. Imipramine treatment increased the levels of the three β-subunit transcripts (β1 by 53%, β2 by 26% and β3 by 39%). Phenelzine treatment significantly altered the levels of β1-subunit mRNA (66% increase), and buspirone-treated animals presented significant decreases in β2- and β3-subunit mRNA levels (34 and 29%, respectively). It can also be observed from Figure 5.2 that the antipanic drugs displayed opposite effects to buspirone on the expression of the β1-subunit gene. However, the effect of alprazolam seemed blunted (18% increase only) compared to the effect produced by imipramine or phenelzine treatment. Similarly, the antipanic agents also produced opposite effects to buspirone on the expression of the β3-subunit gene; however, imipramine was the only drug to produce a significant effect.

Similar to the changes in gene expression of the α- and β-subunit genes, the pattern of alterations in γ-subunit mRNA levels were drug-specific (Figure 5.3). Imipramine treatment significantly increased the expression of γ2-and γ3-subunit genes (81% and 114%, respectively), while phenelzine increased γ2-subunit mRNA levels by 52%. In contrast, buspirone and alprazolam did not produce any significant effects. However, in contrast to buspirone, the antipanic agents seemed to commonly increase the expression of γ2-subunit gene, but the effect of alprazolam (33% increase) was less than that of imipramine and phenelzine. Another common effect of the antipanic drugs on γ1-subunit mRNA seemed to emerge, but none of those increases was significant.

In a similar manner to the results obtained in cortex and cerebellum, the brainstem data were pooled by gene cluster (Figure 5.4). The global effect of buspirone was to reduce GABA$_A$ receptor subunit gene cluster expression. Imipramine had an up-regulating effect on the expression of the three GABA$_A$ receptor subunit gene clusters, that was more pronounced for some subunits. Phenelzine did not induce any coordinated alteration in a specific gene cluster expression since the data are scattered around the null change line. In contrast, alprazolam presented a grouped effect on α2α4β1γ1 gene cluster expression.

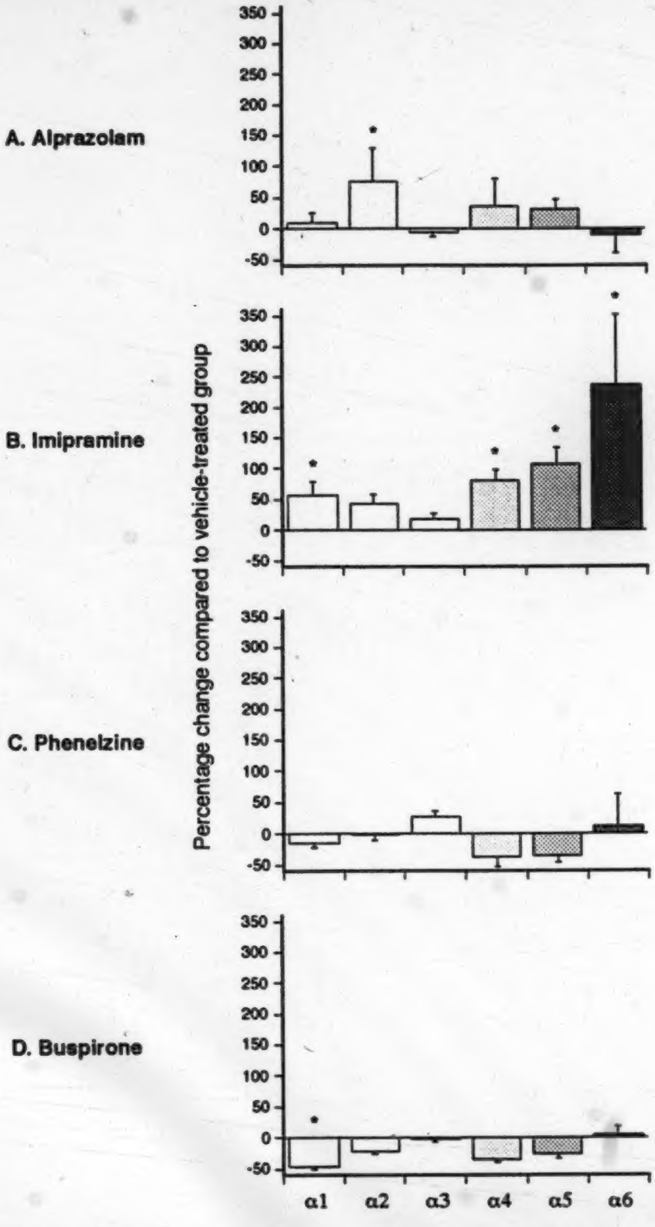

Figure 5.1 Effect of 21 days of drug treatment on brainstem α-subunit gene expression
Data represent the mean ± s.e.m. (n=6) percentage change in the various α-subunit mRNA levels relative to the vehicle-treated group. Statistical analyses were performed by the Dunnett's test pairwise comparison between drug- and vehicle-treated groups, * $P < 0.05$.

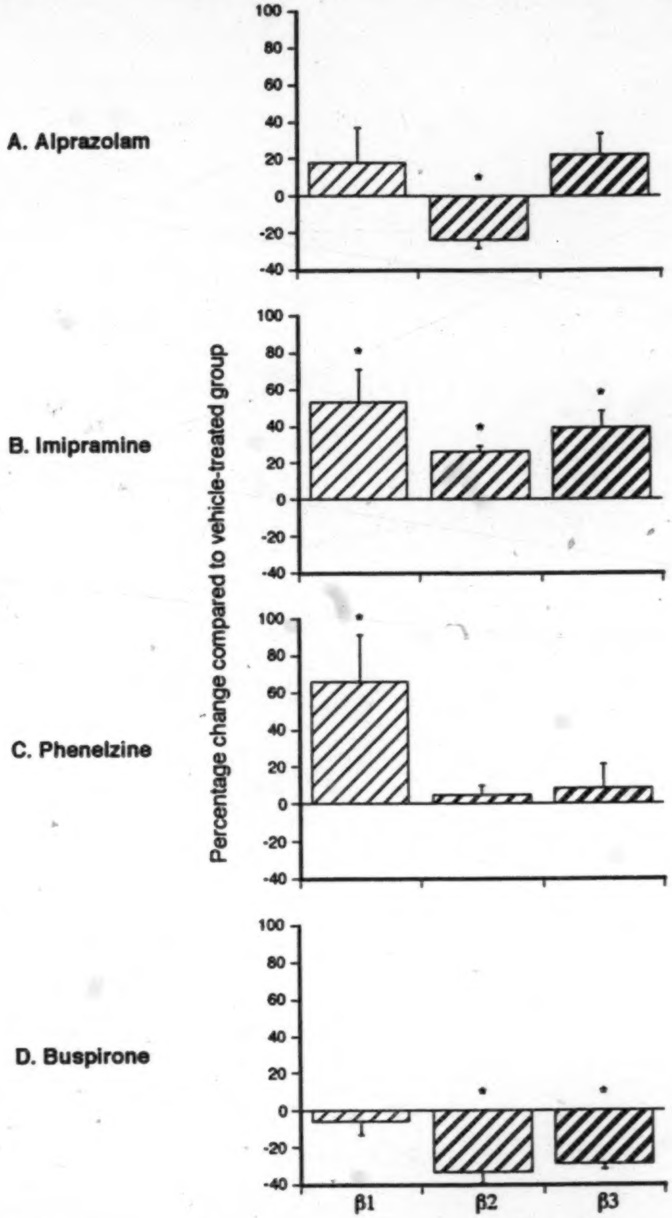

Figure 5.2 Effect of 21 days of drug treatment on brainstem β-subunit gene expression

Data represent the mean ± s.e.m. (n=6) percentage change in the various β-subunit mRNA levels relative to the vehicle-treated group. Statistical analyses were performed by the Dunnett's test pairwise comparison between drug- and vehicle-treated groups, * $P < 0.05$.

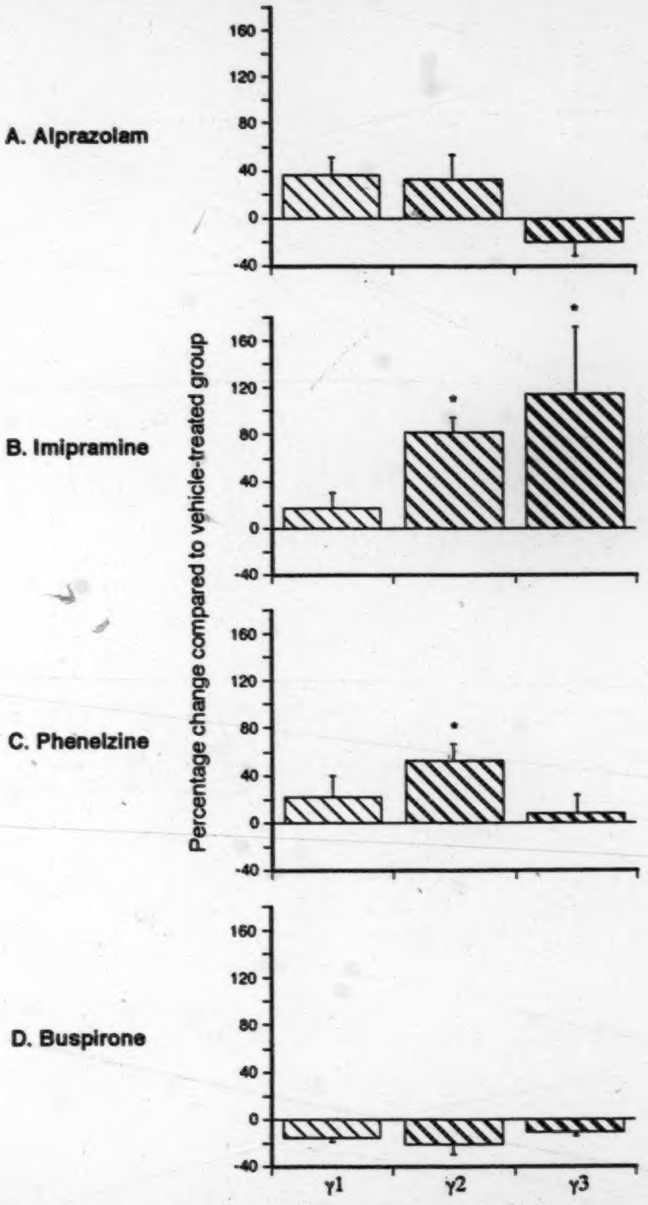

Figure 5.3 Effect of 21 days of drug treatment on brainstem γ-subunit gene expression
Data represent the mean ± s.e.m. (n=6) percentage change in the various γ-subunit mRNA levels relative to the vehicle-treated group. Statistical analyses were performed by the Dunnett's test pairwise comparison between drug- and vehicle-treated groups, * $P < 0.05$.

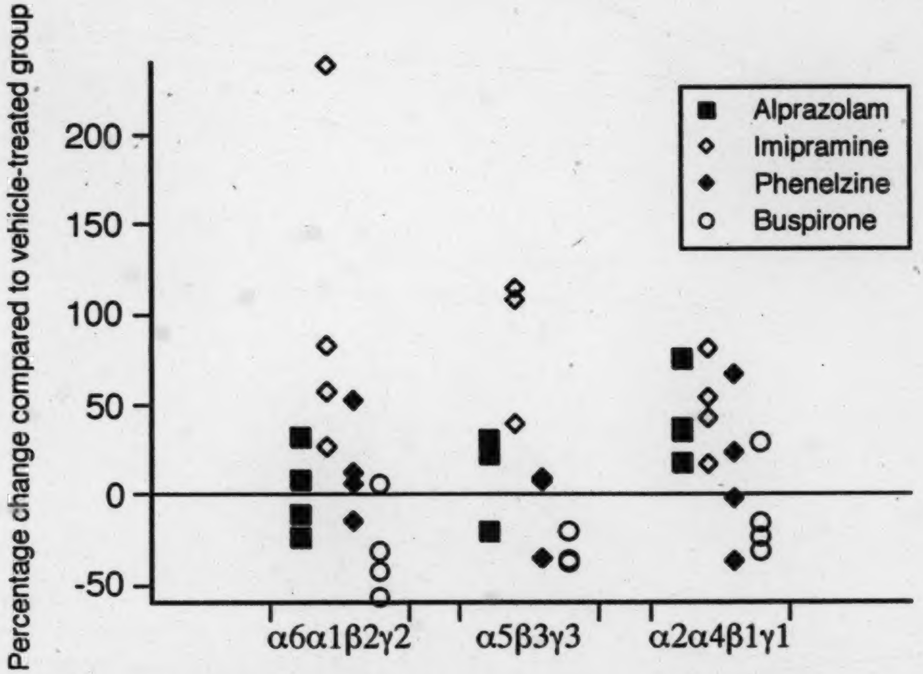

Figure 5.4 Effect of chronic drug treatment on GABA$_A$ receptor subunit gene cluster expression in brainstem
For each drug that induced significant alterations in GABA$_A$ receptor subunit gene expression, the mean percentage changes in subunit mRNA level were grouped by gene cluster. The data represent the individual mean percentage in subunit mRNA levels that correspond to a particular gene cluster.

5.3. DISCUSSION

As indicated before, part of the data presented in this chapter has been previously published (Tanay et al., 1996). However, the reader may realize that some numerical differences exist between the published results (that presented the alterations in α1-, β2- and γ2-subunit mRNA levels) and the data presented in this chapter. Therefore, all factors that may account for these differences will be reviewed.

The data of this chapter represent the pooled results of 2 to 5 replicates (mostly 3-4 replicates) of the experiments, whereas the preliminary data presented the results of a single experiment (n=6). As mentioned in Chapter 3, the decision to replicate the experiments in order to pool the results stemmed from the observation that the multiprobe solution hybridization method presented some inter-experiment variation, the origins of which remain unknown. For this data set, the inter-experiment variation for each treatment x subunit data was such that 3 out of 4 replicate means will be in the range of the pooled mean ± 40.62% of the pooled mean value. Consequently, replicate means comprised within the expected inter-experiment range were considered not different from the pooled mean.

When taking into account the inter-experiment variation, the results presented here are for the most part in agreement with the preliminary data published previously (Tanay et al., 1996) that only examined three subunits. Hence, the effects of phenelzine and buspirone on α1, β2 and γ2 subunits, alprazolam on α1 and β2 subunits and imipramine on α1, β2 and γ2 subunits described in this chapter were not different from the results previously published (Tanay et al., 1996). The amplitude of increase in α1-subunit mRNA level by imipramine reported here (57%) is somewhat lower than in the previous data set (178%), but both are significantly different from control. The principal difference between the two sets of results was a non-significant increase (33%) in γ2-subunit mRNA level after alprazolam treatment which was reported as a significant decrease (44%) in the first experiment.

Factors that may account for these differences include the statistical tests used and the method of analysis. Previously, the data were analyzed with a two-way ANOVA followed the Fisher's least significant difference as post-hoc test, whereas the data presented in this chapter were analyzed with Dunnett's pairwise comparison. The re-analysis of the first experimental result with Dunnett's comparison revealed that the 44% decrease in γ2-subunit mRNA level induced by alprazolam treatment was not significant. In addition, the image of the autoradiogram used to be captured via a video camera, whereas present data were generated by scanning the autoradiograms. The image obtained from the scanner has a greater resolution and presents fewer inter-run variations compared to images captured via the video camera. Hence the scanning the autoradiogram converted the 178 ± 71% increase in α1-subunit after imipramine treatment into a 156 ± 49% increase.

Finally, the alprazolam data presented here come from a different group of rats. A second group of rats was treated with alprazolam for 21 days after suspecting that mRNA degradation may have occurred in the original samples. The degradation of the first group of mRNA samples stemmed from their repetitive usage while determining the specific activities ratio of the various oligonucleotides required for a proper detection in the solution hybridization assay. Analysis of the drug levels in the two groups also revealed that the spinal cord drug concentration of the first group was more heterogeneous than that of the second. The heterogeneity of alprazolam concentrations from the spinal cord of these groups of animals resembles the variation in psychiatric drugs concentrations found in the clinical population. Pharmacogenetic studies have linked this drug level heterogeneity to the occurrence of several metabolic phenotypes characterized by different metabolic abilities. In the present experiment, the mean alprazolam spinal cord concentration for the first group of animals was 37.44 ± 16.25 ng/g ranging from 2.40 to 103.15 ng/g, whereas the second group spinal cord concentration was 23.37 ± 3.87 ng/g with a range from 9.08 to 38.01 ng/g. Hence, the results from the second group are believed be more homogenous. The heterogeneity in the spinal cord alprazolam concentration may be of special importance in light of the results from acute administrations which show that at cortex concentrations of 2-7 ng/g the alprazolam effect on [^{3}H]-Ro 15-1788 binding in some brain regions is opposite to that produced by >20 ng/g levels (Miller et al., 1987). Since Ro 15-1788 binds to all αβγ2 receptor subtypes, these results suggest that the number of αβγ2 receptors is differentially affected by the drug concentration. Regarding the results presented in this chapter, there was no correlation between the levels of γ2-, α2- and β2-subunit mRNAs and the concentration of alprazolam in spinal cord, indicating that the drug levels do not directly influence these subunit gene expressions.

Evidence indicates that brainstem GABA$_A$ receptor heterogeneity in the pons-medulla region is high, due to discrete expression and some unusual combinations of subunits amongst the different nuclei. Immunohistochemistry with subunit-specific antibodies for seven major subunits (α1, α2, α3, α5, β2/3, γ2, δ) was used to determine the distribution of these subunits throughout the brain, although the location of the β2 and β3 subunits could not be distinguished from each other, since the antibody used recognizes both subunit proteins (Fritschy and Mohler, 1995). These experiments revealed that the intense staining of individual brainstem neurons was not apparent at low magnification due to the abundance of white matter (Fritschy and Mohler, 1995). In the pons-medulla regions the predominant GABA$_A$ receptor subunits are α1, α3, β2/3 and γ2 (Fritschy and Mohler, 1995). Indeed, at the pons level, the reticular formation showed an immunoreactivity for α1, α3, β2/3 and γ2 subunits, whereas the auditory pathway in the superior olivary complex presented an uncommon α3-, α5- and γ2-subunit immunoreactivity combination which lacked labeling for α1 and β2/3 subunits (Fritschy and Mohler, 1995). Further, the LC was labeled with α2-, α3- and γ2-subunit specific antibodies but lacked staining with α1-, α5 and β2/3-subunit antibodies (Fritschy and Mohler, 1995). At the medulla level, the reticular formation retained virtually the same immunoreactive staining for α1, α3, β2/3 and γ2 subunits, whereas the inferior olive of the auditory pathway presented immunoreactive staining for α2, α3 and γ2 subunits but lacked staining for α1 and β2/3 subunits (Fritschy and Mohler, 1995). The NTS showed moderate immunoreactive staining for α1, α2, α3, α5, β2/3 and γ2 subunits (Fritschy and Mohler, 1995). Finally, the raphé nuclei were immunoreactive for α1, α2, α3, β2/3 and γ2 subunits (Fritschy and Mohler, 1995), and other studies showed that majority of raphé magnus serotonergic neurons expressed only the α3 subunit and that most GABAergic neurons co-expressed α1 and α3 subunits whereas the remaining GABAergic neurons were stained for α1 subunit only or were devoid of both α1- and α3-subunit immunoreactivity (Gao et al., 1993; Hama et al., 1997).

In situ hybridization experiments investigating the expression of the 13 major subunits (α1-6, β1-3, γ1-3 and δ) revealed that LC neurons contained specific mRNAs encoding for the α3-, α2-, α4-, β3-, β1- and γ1-subunit probes (Luque et al., 1994; Tohyama and Oyamada, 1994), suggesting that LC neurons contain GABA$_A$ receptors composed of α3/2 β1/3 γ1/2 subunits. In the auditory brainstem, α1-subunit mRNA was detected at various levels in all nuclei investigated (Wynne et al., 1995). In contrast, α2- and α3-subunit mRNAs were present in most nuclei but in fewer cells and at lower levels than α1-subunit mRNA (Wynne et al., 1995). The laterodorsal tegmental nucleus was positive for α1-, α3-, α4-, β1-3, γ1- and γ2-subunit transcripts (Tohyama and Oyamada, 1994). The lateral and medial parabrachial nuclei contained mRNAs encoding α1-, α3-, α4-, β1-, β3- and γ2-subunits, but the lateral parabrachial nucleus also included γ1-subunit transcripts (Tohyama and Oyamada, 1994).

Both immunocytochemistry and in situ hybridization studies suggest that the heterogeneity of brainstem GABA$_A$ receptors is due to the discrete differential expression of subunits both between and within the brainstem nuclei. However, there are some discrepancies between the location of subunit proteins and mRNAs. These are probably due to the fact that the mRNA species mainly co-localize with the cell bodies, whereas the detection of protein indicates where the receptors are present on the neurons (this can include the dendrites and axon terminals which can be remote from the cell body). Therefore, the identification of some mRNA species in a brain structure may correspond to the presence of the corresponding proteins in other nuclei.

The pattern of changes in GABA$_A$ receptor subunit gene expression reported in this data set is drug- and brain region-specific. Indeed, in contrast to the effect observed in the cortex where buspirone increased GABA$_A$ receptor subunit mRNA levels, this drug decreased the overall expression of the GABA$_A$ receptor subunit gene in the brainstem. Imipramine, which did not induce any changes in the amount of GABA$_A$ receptor subunit steady-state mRNAs in the cortex, increased the levels of these transcripts in the brainstem. In addition, the data indicated that both phenelzine and imipramine increased β1- and γ2-subunit steady-state mRNA levels, whereas alprazolam treatment induced discrete but significant changes on α2- and β2-subunit gene expression.

The analysis of the effects of these drugs on the different GABA$_A$ receptor subunit gene clusters did not unveil any antipanic drug-specific changes. Indeed, imipramine had a positive coordinated effect on all the clusters, but there was a greater spread for the $\alpha6\alpha1\beta2\gamma2$ gene cluster compared to the other two clusters. Phenelzine produced few discrete significant changes in subunit mRNA levels that did not translate into cluster-targeted effects, whereas alprazolam which also induced few significant alterations in GABA$_A$ receptor subunit gene expression, presented a coordinated effect on the $\alpha2\alpha4\beta1\gamma1$ gene cluster similar to that of imipramine. Finally, the decreases induced by buspirone were equally spread on the different gene clusters, although the scatter of the changes induced was tighter for the $\alpha5\beta3\gamma3$ gene cluster.

The alterations in gene expression induced by alprazolam treatment did not match the significant changes induced by imipramine and phenelzine treatments. The antipanic drugs did not seem to produce any common effect on GABA$_A$ receptor subunit gene expression. However, the effects of alprazolam on β1- and γ2-subunit mRNA levels seemed blunted compared to the effects of imipramine and phenelzine, and intermediate to that produced by buspirone. Increases in the levels of mRNA species encoding the β1 and γ2 subunits may be of particular importance in the brainstem. As mentioned above, evidence indicates that both subunits and mRNAs are present in brainstem nuclei, although the distribution of γ2 subunit seems to be more widespread. These results suggest that brainstem GABA$_A$ receptor subunit composition is heterogeneous. In addition, the steady-state mRNA levels have been measured in brain regions, so alterations of a specific mRNA level in a particular brain nucleus may be partially or totally masked by no, or opposite, changes in other structures. Such a situation may give rise to overall no change or small not significant changes similar to those observed for β1-and γ2-subunit mRNAs in alprazolam-treated animals.

Assuming that increases in the expression of β1- and γ2-subunit genes underlie a common antipanic effect, the physiological relevance of such increases relative to the possible receptor subtypes found in the brainstem is of great interest. Electrophysiological studies on recombinant GABA$_A$ receptors expressed in Xenopus oocytes or HEK 293 cells provide insight into the possible functional consequences of the transfection of different β- or γ-subunit isoforms on GABA-gated currents. In the case of a replacement of the β2 by the β1 subunit in a given αγ2 combination, the EC$_{50}$ of GABA for the recombinant receptor does not seem affected (Ebert et al., 1994; Ducic et al., 1995), except for the α3γ2 subunit combination which displays a marked reduction in affinity for GABA (Sigel et al., 1990; Ebert et al., 1994). In addition, for a given αγ2 subunit combination, the maximal current amplitude elicited by GABA is decreased by 10 to 60% in β1- compared to β2-subunit containing receptors, with the exception of the α6γ2 subunit combination which displays a 60% current increase (Ducic et al., 1995). In contrast, the replacement of the β3 by the β1 subunit changes the EC$_{50}$ of the recombinant receptors to GABA (Ebert et al., 1994; Ducic et al., 1995). Similarly, the presence of the γ2 subunit in place of the γ1 or γ3 subunit increases the EC$_{50}$ of the receptor for GABA and the maximal amplitude of GABA-elicited currents in α1β2, α1β1 or α3β1 subunit receptor combinations (Ducic et al., 1995; Ebert et al., 1994). Thus, β1 and γ2 subunits influence GABA sensitivity and maximal effect of the resulting receptor subtype. Finally, evidence suggests that the α-subunit isoform also plays a crucial role in the determination of the sensitivity of the receptor for GABA (Ducic et al., 1995; Ebert et al., 1994; Sigel et al., 1990). GABA$_A$ receptor subtypes containing the α3 subunit, especially those that also include β1 and γ2 subunits, display an increased GABA EC$_{50}$ and a lower GABA-gated maximal current amplitude compared to their α1-, α5 or α6-subunit containing counterparts (Ebert et al., 1994; Sigel et al., 1990; Sigel et al., 1992; Wafford et al., 1993; Malherbe et al., 1990; Ducic et al., 1995). In combination with the β2γ2 subunit complex, α3-subunit containing receptors present a decrease in GABA EC$_{50}$ without changes in GABA-gated maximal current amplitude (Ducic et al., 1995; Ebert et al., 1994). Finally, the upregulation of α3β1γ2 receptor subtypes at a particular synapse is expected to profoundly affect the channel response to GABA, and therefore the GABA-mediated inhibitory tone.

To summarize, no definite common changes in $GABA_A$ receptor subunit gene expression was uncovered in the brainstem after chronic treatment with the antipanic drugs. If alprazolam induces alterations in the β1- and γ2-subunit gene expression similar to those triggered by imipramine and phenelzine treatments in identical brain regions, then these subunit genes are the best candidates for being the molecules commonly affected by antipanic drugs. Indeed, the upregulation of β1- and γ2-subunit gene expression after chronic treatment with antipanic drugs may correspond to a switch in the receptor subunit composition within particular brainstem nuclei and receptors. Such subunit substitution is expected to give rise to receptors with altered sensitivity and response to GABA, which in turn may alter the inhibitory effects of GABA on the targeted receptors and the GABAergic regulatory tone on a particular brain structure.

6. Further examination of the brainstem: a key brain region for antipanic drug action?

6.1. INTRODUCTION

6.1.1. Antipanic drugs and α3-, β1- and γ2-subunit gene expression

The results presented in Chapter 5 indicated that 21 day treatment with phenelzine or imipramine significantly increased brainstem $GABA_A$ receptor γ2- and β1-subunit gene expression. In addition, compared to buspirone, both drug treatments caused a small rise in α3-subunit mRNA levels. Alprazolam altered β1- and γ2-subunit gene expression in a manner similar to that of imipramine and phenelzine, but the amplitude of these changes was somewhat blunted. As previously discussed (Chapter 5), $GABA_A$ receptor subunit genes are differentially expressed in discrete brainstem nuclei and the subunit distribution is heterogeneous between different cell types within a given nucleus (Fritschy and Mohler, 1995; Gao et al., 1993; Hama et al., 1997). Hence, the changes in β1- and γ2-subunit gene expression in a specific cell type within a discrete brain nucleus brought on by long-term treatment with alprazolam may not be seen in these experiments if concomitant with opposite or no alterations in other areas. Thus, it is possible that in restricted brainstem structures, alprazolam administration alters these subunit mRNA levels in a similar manner to that of imipramine and phenelzine. However, since alprazolam is both an anxiolytic and an antipanic agent, several questions arise. Is the brainstem the key brain region for an antipanic therapeutic effect? Do the alterations in α3-, β1- and γ2-subunit gene expression reflect an antidepressant or an antipanic effect? $GABA_A$ receptor ligands induce differential time-dependent changes in the expression of these receptor subunit genes (Heninger et al., 1990; Kang and Miller, 1991; O'Donovan et al., 1992; Brown and Bristow, 1996; Holt et al., 1996; Holt et al., 1997; Holt, 1998; Wu et al., 1995; Calkin and Barnes, 1994; Zhao et al., 1995). Therefore, is 21 days the best time point to detect alterations induced by alprazolam treatment? The clinical data clearly indicate that chronic administration is required for the onset of antipanic effect of imipramine and phenelzine (Ballenger, 1986; Ballenger, 1991; Schweizer et al., 1993; Black et al., 1993; Burrows et al., 1993; Salzman, 1993; Pecknold, 1993; Curtis et al., 1993; Rosenberg, 1993; Layton and Dager, 1994; Westenberg, 1995; Westenberg, 1996; Milrod and Busch, 1996). Hence, any alteration in gene expression that correlate with the antipanic activity of these drugs should not be present after short-term drug administration. Consequently, are the changes in gene expression induced by imipramine and phenelzine specifically occurring upon chronic administration?

As mentioned in Chapter 1, the treatment of PD patients with tricyclic and serotonergic antidepressants is characterized by an initial worsening of the symptoms before an improvement occurs (Ballenger, 1991; Layton and Dager, 1994). In contrast, the onset of benzodiazepine antipanic therapy seems faster than that of the antidepressants (Sheehan et al., 1990; Alexander and Alexander, 1986; Charney et al., 1986; Ballenger et al., 1988; Schweizer et al., 1993; Davidson, 1997; Bradwejn, 1993a; Ballenger, 1991). Today's therapeutic approach is to start patients with a co-treatment of a benzodiazepine and an antidepressant, and to gradually taper the benzodiazepine after the onset of the therapeutic effect (Bradwejn, 1993a; Rosenberg, 1993; Layton and Dager, 1994). This clinical practice suggests that a 21 day treatment period of experimental animals may not be the most appropriate time point to determine the effects of alprazolam on $GABA_A$ receptor subunit gene expression. If indeed alterations in α3-, β1- and γ2-subunit mRNA levels underlie the onset of antipanic therapeutic effect, then the maximal changes in gene expression common to these drugs may be transient and could also occur at an earlier time point for alprazolam. Consequently, the determination of alterations in these subunit mRNA levels after 21 days of treatment may be beyond the time point of maximal changes for alprazolam. Indeed, time point studies of the effect of diazepam and zolpidem on $GABA_A$ receptor subunit gene expression have shown that these drugs induce maximal changes in some subunit mRNA levels after 7 days of treatment, and that these changes wane over time (Holt et al., 1996; Holt et al., 1997). To determine whether α3-, β1- and γ2-subunits may be another possible target for antipanic agents, the effect of alprazolam on these subunits' gene expression was carried out after treatment for 14 days.

As reviewed in Chapter 1, alprazolam, imipramine and phenelzine increase aspects of $GABA_A$ receptor-mediated neurotransmission. In addition, as stated above, $GABA_A$ receptor ligands induce time-dependent changes in subunit gene expression. Consequently, if changes in α3-, β1- and γ2-subunit mRNA levels underlie the onset of antipanic therapeutic effect, the maximal changes in gene expression common to the antipanic drugs may be temporary and occur only upon long-term administration of these drugs. In order to determine whether antipanic drug-induced alterations of $GABA_A$ receptor subunit gene expression are related to a long-term treatment, the chronic effects of these drugs on α3-, β1- and γ2-subunit mRNA were compared to those of a short-term administration (3 days).

6.1.2. Antipanic drugs, CCK transmission and $GABA_B$ receptors

In addition to GABA, CCK is another neurotransmitter of special interest in the brainstem with respect to PD. As discussed in Chapter 1, CCK is a recognized panicogenic agent and is frequently co-localized with GABA. The CCK gene encodes a prepropeptide which is cleaved to generate several peptides that are distributed in a region-dependent manner in the brain (Rehfeld and Nielsen, 1995). CCK peptides act at 2 types of G-protein coupled receptors, CCK_A and CCK_B, and an hypersensitivity of CCK_B receptors has been suggested in PD patients (Akiyoshi et al., 1996; Akiyoshi et al., 1997).

Physiological interactions between GABAergic and CCK neurotransmission have been shown in different brain areas, and both presynaptic and postsynaptic interactions seem to occur. Indeed, evidence suggests that, presynaptically, GABA can modulate CCK release via $GABA_A$ receptor-mediated mechanisms (Abucham and Reichlin, 1991; Benoliel et al., 1992; Nevo et al., 1996; Harro et al., 1993). Similarly, CCK modulation of GABA release via CCK_B-receptor mediated mechanisms seems to involve CCK_B receptors located on GABAergic interneurons (Perez de la Mora et al., 1993; Albrecht et al., 1995; Rakovska, 1995). Further, at least in the solitary complex, endogenous CCK acting at CCK_B receptors has been reported to decrease the amplitude of $GABA_A$ receptor-mediated inhibitory transmission from GABAergic interneurons (Branchereau et al., 1992b). Accordingly, the potentiation of the inhibitory effects of exogenously applied GABA by the CCK_B receptor antagonist, PD 134,308, has been suggested to involve a cytosolic mechanism (McLean et al., 1996). In addition, evidence suggests that benzodiazepine site ligands modulate the level of expression of the CCK gene (Rattray et al., 1993; Pratt and Brett, 1995) and $[^3H]$-CCK_8 binding site density (Harro et al., 1990). Finally, other classes of drugs have also been reported to alter CCK levels in rat brain (Frey, 1983). Therefore, it is possible that the long-term effects of antipanic drugs also involve a modulation of CCK system gene expression, as a means to re-balance GABAergic-CCK neurotransmission interactions. Preliminary experiments investigating the long-term alterations of CCK and CCK receptor gene expressions induced by antipanic agents were performed. CCK, CCK_A receptor and CCK_B receptor transcript levels were determined after 14 days, or 21 days of treatment with alprazolam, or the antidepressants, respectively. These time points for treatment were chosen based on the speculation that the effect of alprazolam on mRNA levels (reflecting the faster onset of action of this drug) occurs at an earlier time point compared to imipramine and phenelzine.

Finally, $GABA_B$ receptors are responsible for the slow synaptic inhibition mediated by GABA (Kerr and Ong, 1995). Presynaptically, these G-protein coupled receptors are also involved in the regulation of GABA release (Kerr and Ong, 1995). At present, little evidence supports the primary involvement of these receptors in PD, since the GABA$_B$ receptor agonist, baclofen, displayed antipanic efficacy in only one open study (Breslow et al., 1989). In addition, chronic treatments with imipramine or phenelzine had no effect GABA$_B$ receptor binding in the cortex (McManus and Greenshaw, 1991; Engelbrecht et al., 1994). However, recent evidence supports the functional and molecular heterogeneity of these receptors (Kerr and Ong, 1995; Kaupmann et al., 1997). Despite both GABA$_B$ receptor subtype transcripts being absent from glial cells, the relative neuronal distribution and pharmacological specificity of the two GABA$_B$ receptor subtypes are not established (Kaupmann et al., 1997). Thus, so far there is no established link between a particular GABA$_B$ receptor subtype and specific functional or pharmacological properties, suggesting that, as for GABA$_A$ receptors, the current pharmacological tools may limit the investigations of these receptors. The possible contribution of these receptors to the GABAergic-CCK neurotransmission balance is supported by the fact that K$^+$-stimulated release of CCK-like immunoreactivity from cortical synaptosomes or striatal slices is decreased by GABA$_B$ receptor agonists (Raiteri et al., 1993; Raiteri et al., 1996; Gemignani et al., 1994). In the spinal cord, the GABA$_B$ receptor agonist, baclofen, regulates CCK-like immunoreactivity release (Benoliel et al., 1992). Hence, as intrinsic elements of the GABAergic transmission involved in the regulation of GABA release, and as possible factors in the interaction between GABA and CCK systems, GABA$_B$ receptors may also be implicated in the efficacy of antipanic drugs. Similar to the CCK system parameters, a preliminary investigation of the effect of chronic treatment with imipramine, phenelzine or alprazolam on GABA$_B$ receptor mRNA levels was completed. Assuming that the time scale of the occurrence of the variations in mRNA levels is similar to that of the CCK system genes, the effects of chronic treatment with antipanic agents on GABA$_B$ receptor gene expression were determined after 21 days administration of imipramine or phenelzine, and after 14 days infusion of alprazolam

To summarize, this chapter describes the successive investigations of the effects of antipanic agents on selected GABA$_A$ receptor subunit transcripts levels, and on CCK, GABA$_B$ and CCK receptors gene expression. Firstly, It was determined whether antipanic agents produce common alterations in GABA$_A$ receptor α3-, β1-, and γ2-subunit gene expression. To this aim, the effects of alprazolam on these subunit mRNA levels were measured at an earlier time point (14 day treatment), and compared to those of 3 week treatment with imipramine, phenelzine or buspirone. In addition, in order to establish that the changes in gene expression were specific to long-term drug delivery, the effects of alprazolam, imipramine, phenelzine and buspirone on α3- β1- and γ2-subunit gene expression were determined after 3 days of treatment. Secondly, a preliminary set of experiments was conducted to investigate whether antipanic drugs action could involve an alteration of the GABA/CCK neurotransmission balance. The distribution of CCK_A and CCK_B binding sites in the brain has been extensively studied (Mercer et al., 1996; Mercer and Beart, 1997; Hill et al., 1990), but similar to $GABA_B$ receptor isoforms, the distribution of the different molecular forms of CCK_B receptors and CCK remains unknown. Therefore, the oligonucleotide probes used in this study were designed to recognize mRNAs that encode both $GABA_B$ receptor isoforms, the untruncated CCK_B receptor, CCK_A receptor, and CCK.

6.2. RESULTS

6.2.1. Effect of 14 days of treatment with alprazolam on α3-, β1- and γ2-subunit gene expression

The effects of 14 day treatment with alprazolam presented in Figure 6.1 are the pooled results of 2 separate experiments using alprazolam- (n=6) and vehicle-treated (n=3) animals. The data were combined with those from imipramine, phenelzine and buspirone-treated groups (21 days) to retain the same number of degrees of freedom, and keep the statistical analysis with Dunnett's pairwise comparison to the pooled vehicle group consistent. Surprisingly, 14 day treatment with alprazolam does not significantly alter α3-, β1- and γ2-subunit mRNA levels. The changes induced after 14 days of treatment are almost identical to those observed after 21 day treatment (Figures 5.1, 5.2 and 5.3). Finally, the analysis of the 27% increase in α3-subunit mRNA level induced by chronic phenelzine treatment, which was previously reported in Chapter 5 (Figure 5.1) as non-significant, reached the 5% level of significance.

6.2.2. Effects of 3 days of treatment on $GABA_A$ receptor α3-, β1- and γ2-subunit gene expression

The variations in α3-, β1- and γ2-subunit transcript levels after 3 day administration of the different drugs is presented in Figure 6.2. The data presented are the mean of 3 separate (alprazolam) or 2 separate (imipramine, phenelzine and buspirone) experiments. Short-term treatment with alprazolam significantly increases α3-, β1- and γ2-subunit gene expression by 32%, 72% and 143% respectively, whereas imipramine, phenelzine and buspirone are devoid of any significant effects.

6.2.3. Effects of chronic drug treatments on the gene expression of CCK, GABA$_B$ receptor and CCK receptors

In the brainstem, GABA$_B$ receptor genes are highly expressed, whereas CCK$_B$ receptor and CCK transcripts are present at intermediate levels, and CCK$_A$ receptor mRNA is present at very low levels. The effect of 14 day administration of alprazolam and 21 day treatment with imipramine, phenelzine or buspirone are shown in Figure 6.3. The data presented are the means of two separate experiments for each group of samples. None of these drugs produced any effect on either CCK$_A$ or CCK$_B$ receptor gene expression. However, alprazolam significantly increased the levels of mRNA encoding for GABA$_B$ receptor and CCK (53% and 63%, respectively), whereas phenelzine significantly decreased CCK mRNA levels by 46%.

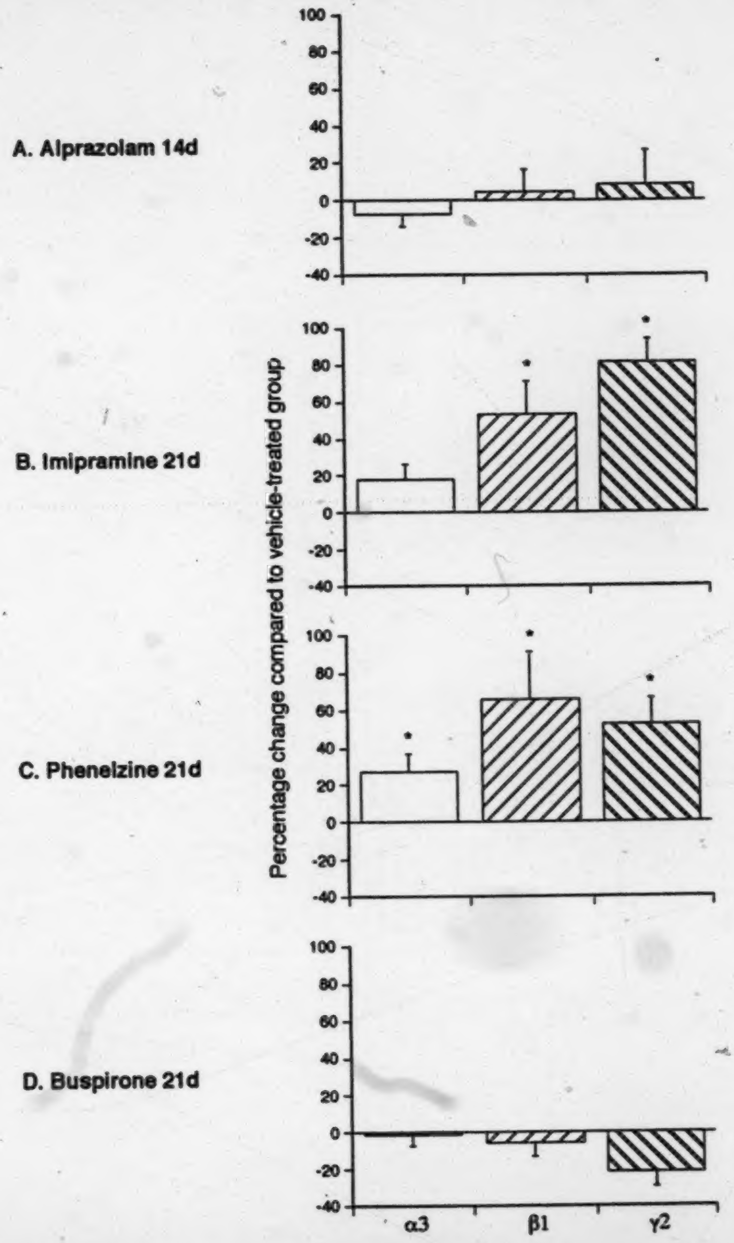

Figure 6.1 Changes in α3- β1- and γ2-subunit mRNA levels after chronic treatment with either alprazolam (14 days) or imipramine, phenelzine or buspirone (21 days) Data represent the mean ± s.e.m. (n=6) percentage change in α3- β1- and γ2-subunit transcript abundance relative to the vehicle-treated group. Statistical analyses were performed by Dunnett's test pairwise comparison between drug- and vehicle-treated group, *$P<0.05$.

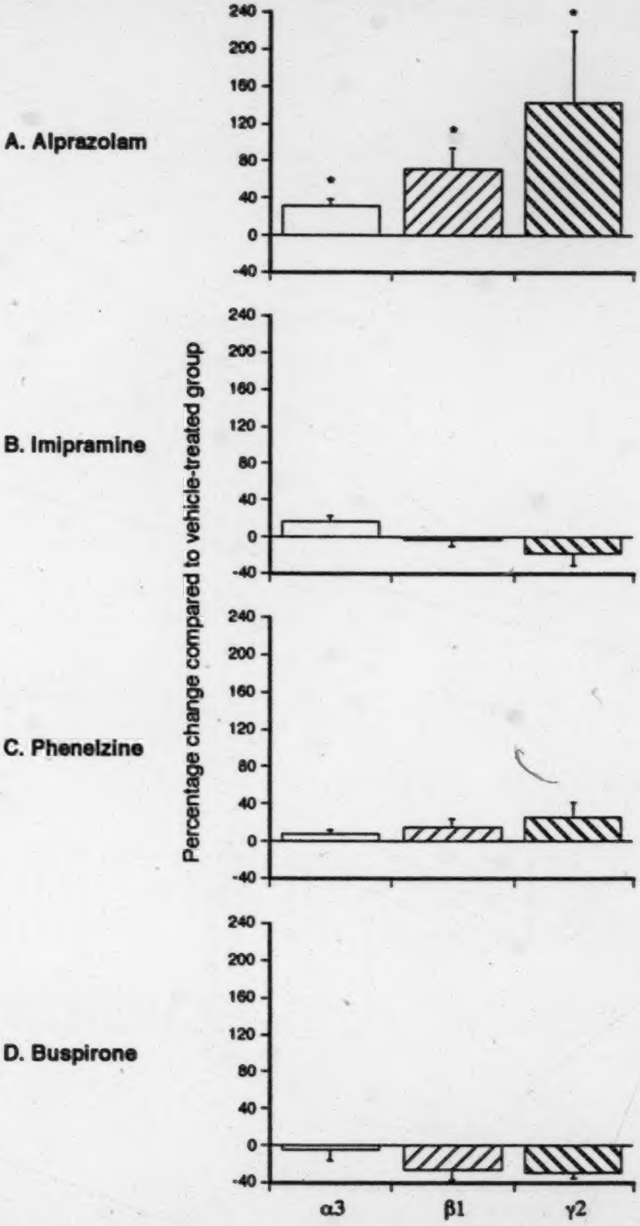

Figure 6.2 Effect of 3 days of drug treatment on α3- β1- and γ2-subunit steady state mRNA levels

Data represent the mean ± s.e.m. (n=6) percentage change in α3- β1- and γ2-subunit transcript abundance relative to the vehicle-treated group. Statistical analyses were performed by Dunnett's test pairwise comparison between drug- and vehicle-treated group, **P*<0.05.

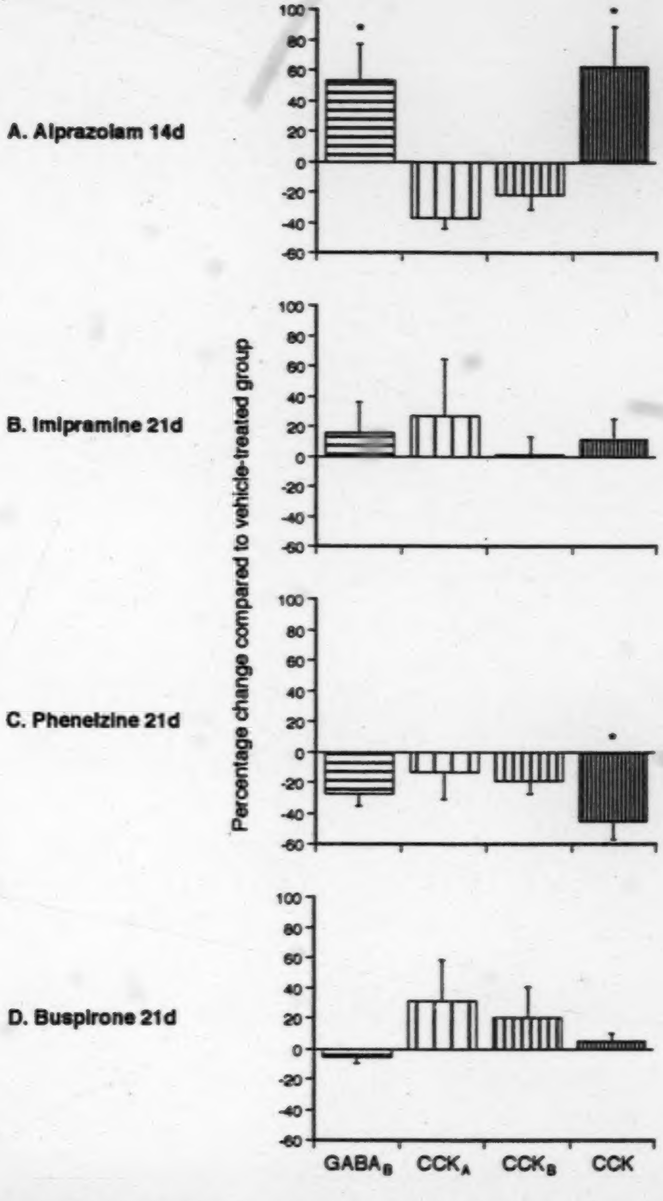

Figure 6.3 Effect of chronic drug treatment on GABA$_B$, CCK$_A$ and CCK$_B$ receptors and CCK steady-state mRNA levels

Data represent the mean ± s.e.m. (n=6) percentage change in GABA$_B$ receptor, CCK$_A$ receptor, CCK$_B$ receptor and CCK transcripts relative to the vehicle-treated group. Statistical analyses were performed by Dunnett's test pairwise comparison between drug- and vehicle-treated group, $^{*}P<0.05$.

6.3. DISCUSSION

The results reported here show that 14 day administration of alprazolam did not change the levels of α3- β1- and γ2-subunit transcripts. However, a 3 day treatment with this drug induced variations in the steady-state levels of α3- β1- and γ2-subunit mRNA. The alterations induced by short-term alprazolam administration paralleled the variations induced by 21 day imipramine or phenelzine treatment, and the amplitude of changes in α3- and β1-subunit mRNAs are similar for the three drugs. The short-term administration of alprazolam produced a mean increase of 143% in γ2-subunit mRNA levels, but the group contained an outlier (511% increase) which could not be excluded since its value was within 2 standard deviations of the mean (standard deviation of 189%). However, if that data point was excluded, the mean rise in γ2-subunit mRNA levels (70%) induced by 3 day alprazolam treatment would fall within the mean increases caused by either imipramine or phenelzine chronic administration (81% and 52%, respectively). In addition, the 32% mean increase in α3-subunit mRNA levels produced after short-term alprazolam administration is close to the mean changes induced by 21 day treatment with imipramine (18%) and phenelzine (27%). It should be noticed, however, that the inclusion of the 14 days of alprazolam treatment data set changed the statistical significance of the alterations induced by 21 days administration of phenelzine (Figure 5.1). Indeed, the 27% increase in α3-subunit mRNA levels after 21 days of administration of phenelzine now reached the 5% level of significance (Figure 6.1). Since the mean and the variance on the α3-subunit mRNA levels of the alprazolam-treated group are almost identical for the two time points (mean: -7.12%, variance: 0.018% at 21 days; mean: -7.12%, variance: 0.025% at 14 days), the explanation of such a result is that the variance of the α3-subunit mRNA level of the vehicle group for the 14 day study (0.005%) was much smaller than for the 21 days of treatment data set (0.156%). Therefore the mean error sum of squares calculated for the ANOVA decreased, and consequently the *F* value increased. In addition to the α3 subunit, the administration of alprazolam for 3 days enhanced the expression of β1-subunit gene by 72%, while 21 day of treatment with imipramine or phenelzine augmented this subunit mRNA levels by 53% and 66% respectively. Finally, the effects of buspirone on α3- β1- and γ2-subunit transcript levels varied from 1%, 6% and 22% decrease after 21 days of treatment to 4%, 26% and 28% decrease after 3 day of administration, respectively.

In contrast to buspirone, the antipanic drugs alprazolam, imipramine and phenelzine produced common changes in α3- β1- and γ2-subunit gene expression in the brainstem. However, their effects were on a different time scale to each other. The effects of imipramine and phenelzine required longer administration (21 days) than alprazolam (3 days). It is possible that the changes in α3- β1- and γ2-subunit gene expression may be related to the therapeutic onset of antipanic agents. Compared to the antidepressants, the benzodiazepines display an earlier onset of antipanic action (Alexander and Alexander, 1986; Charney et al., 1986; Ballenger et al., 1988; Schweizer et al., 1993; Davidson, 1997; Ballenger, 1991; Bradwejn, 1993a). Thus, alprazolam's faster onset of action could be concomitant or subsequent to the changes in α3- β1- and γ2-subunit mRNA levels which occur after 3 days of treatment, whereas imipramine's and phenelzine's delayed therapeutic onset may be due to the changes in gene expression occurring after a longer period.

According to Ballenger (Ballenger, 1991), the long-term pharmacological treatment of PD can be modeled on the succession of three phases: acute, stabilization and maintenance. This model proposes that the acute phase corresponds to the onset of the antipanic effect (reduction of symptoms). In contrast, the stabilization and the maintenance phases aim at the extension of the therapeutic effects of antipanic agents to other aspects of the disorder and sustain the improvement to allow a return to normal life. Hence, the confirmation of a relationship between the changes in α3- β1- and γ2-subunit transcript levels and the onset of antipanic effect would support this model and that alprazolam's onset of action is much earlier than that of imipramine or phenelzine (Ballenger, 1991). This relationship may also provide a molecular basis for the onset of the antipanic effect of these drugs. Finally, the results from the time study of the changes in α3- β1- and γ2-subunit mRNAs induced by alprazolam indicated that the alterations observed at 3 days are transient, since their values return to control levels by 14 days of administration. Therefore, if these alterations in gene expression are related to the antipanic action of alprazolam, imipramine or phenelzine, it is expected that these changes would follow similar courses for each treatment but shifted in time. The time course study of the alterations in α3- β1- and γ2-subunit mRNA levels for all the drugs would enable to establish the time scale of occurrence of these changes. Since it is postulated that the transitory changes in α3-, β1- and γ2-subunit mRNA levels underlie the onset of therapeutic action, several molecular events linked to the subsequent therapeutic phases can be considered. The transient effect on α3-, β1- and γ2-subunit transcript levels can be followed by a long-lasting upregulation of the corresponding subunit proteins, and/or can trigger sustained molecular events in the brainstem nuclei carrying these changes in gene expression and/or in the brain areas to which they project.

The effect of antipanic drugs on GABA_A receptor α3- β1- and γ2-subunit mRNA levels suggests that these drugs may increase the occurrence of the α3β1γ2 GABA_A receptor subtype. Evidence suggests that this receptor subtype may naturally occur in the brainstem. *In situ* hybridization experiments have shown that LC neurons contained α3- and β1-, but no γ2-subunit transcripts (Luque et al., 1994; Araki and Tohyama, 1992; Tohyama and Oyamada, 1994), suggesting that the α3β1 subunit combination may be normally expressed by these neurons. The α3 subunit is widely distributed throughout the brainstem, but the highest levels of this subunit mRNA are found in the NTS, inferior olive, trigeminal nuclei, dorsal raphé, parabrachial nuclei, vestibular nucleus and hypoglossal nucleus (Araki and Tohyama, 1992). Other nuclei positive for the α3-subunit transcript include the gigantocellular nuclei, the reticular formation and the laterodorsal tegmental nuclei (Tohyama and Oyamada, 1994; Araki and Tohyama, 1992). In contrast, although the γ2 subunit is the most abundant and widely distributed γ subunit in the brain, the distribution of this subunit is heterogeneous in the brainstem. Indeed, in situ hybridization studies showed that the γ2-subunit mRNA is present in the reticular formation and raphé nuclei, but not in the NTS and the LC (Araki et al., 1992; Luque et al., 1994; Tohyama and Oyamada, 1994). Other nuclei positive for γ2-subunit transcript include the superior and inferior colliculus, the vestibular nucleus, the ventral part of the motor trigeminal and the facial nuclei (Miralles et al., 1994; Araki et al., 1992) the auditory system, the gigantocellular nuclei, the pontine nuclei, the laterodorsal tegmental nucleus, the parabrachial nuclei and the nucleus prepositus hypoglossi (Tohyama and Oyamada, 1994; Araki and Tohyama, 1992). The distribution of the β1-subunit mRNA is restricted to discrete areas of the brainstem, and is at best of moderate intensity. Studies using *in situ* hybridization revealed the presence of the β1-subunit transcript in the central gray, LC and NTS, whereas a weak to moderate signal was observed in the tegmental nuclei, raphé nuclei, pontine nuclei, parabrachial nuclei and gigantocellular nuclei (Tohyama and Oyamada, 1994; Araki and Tohyama, 1992; Luque et al., 1994). Altogether, *in situ* hybridization studies suggest that α3-, β1- and γ2-subunit mRNAs are present in the tegmental, the parabrachial and the gigantocellular nuclei neurons.

Immunohistochemical studies with α3- and γ2-subunit-specific antibodies have, however, revealed the presence of the α3γ2 subunit combination in the reticular formation, auditory pathway, LC, NTS, and raphé (Fritschy and Mohler, 1995). Hence, some of these nuclei, such as the NTS and LC, are immunopositive for a subunit (γ2) but do not contain the corresponding mRNA. Such a discrepancy between the protein and the mRNA levels may be explained by differences in the sensitivity of the detection methods (one-to-one detection for in situ mRNA hybridization versus amplification with secondary or tertiary antibodies for immunocytochemistry), or by an mRNA turnover too fast to allow a detection at steady-state, or by afferent projections from neuronal cell bodies located in different brain structures expressing the corresponding gene that project onto the neuron cell bodies which do not contain the particular subunit. The knowledge of location of the neuronal projections sent by a particular brain structure will enable extrapolation to the location of the functional alterations subsequent to changes in gene expression in this brain area. As summarized above, in situ hybridization studies have shown that α3-, β1- and γ2-subunit mRNAs are present in the tegmental, the parabrachial and the gigantocellular nuclei neurons. The results described in this chapter indicate that antipanic agents might increase the occurrence of the α3β1γ2 $GABA_A$ receptor subtype. If future studies show that the increase in α3-, β1- and γ2-subunit mRNAs observed after the administration of antipanic drugs is located in these brain structures, functional changes might be expected in other areas, such as the LC, to which the gigantocellular nuclei neurons project.

An increase in the level of the α3β1γ2 $GABA_A$ receptor subtype would probably dramatically alter the GABAergic transmission tone at a particular synapse. Evidence suggests that α-subunit distribution is heterogeneous between neuronal populations. Indeed, raphé serotonergic neurons express only the α3 subunit, whereas GABAergic neurons can express either α1 and α3 subunits in combination, only the α1 subunit, or none of these subunits (Gao et al., 1993; Hama et al., 1997). Therefore, an increase in α3-subunit mRNA levels in the raphé may correspond to either a net increase in the α3-subunit containing receptor number, or a switch between the α1- and the α3-subunit containing receptor populations. Further, previous data have shown that α3β1γ2 $GABA_A$ receptors display decreased GABA-gated maximal current amplitudes and a higher GABA EC_{50} compared to many other receptor subtypes (Ducic et al., 1995; Ebert et al., 1994; Malherbe et al., 1990; Sigel et al., 1990; Sigel et al., 1992; Wafford et al., 1993). Hence, the substitution of a given receptor subtype for the α3β1γ2 subunit combination may decrease the GABAergic inhibition at this particular synapse. In contrast, a rise in the total number of $GABA_A$ receptors by increasing the level of the α3β1γ2 receptor subtype may enhance the GABAergic inhibitory tone. The physiological consequence of such a remodeling of GABAergic transmission may be varied. Following the example described in the previous paragraph of the gigantocellular nuclei neurons projecting to the LC, two main scenarios are presented. The substitution of a given $GABA_A$ receptor subtype for the α3β1γ2 subunit combination on these projections would decrease the

GABAergic inhibition of these neurons, which would in turn enhance their excitatory activity on LC neurons. Alternatively, an increase in the total number of $GABA_A$ receptors present on these projections could lead to a reduction in the firing of these neurons. In this particular example, it is expected that the second scenario would take place for PD therapy. However, depending on the pre- an postsynaptic neurons this may not always be the case.

No common effect of the antipanic drugs seemed to arise from the study of the expression of the genes for CCK, $GABA_B$ receptor and CCK receptors, especially since phenelzine and imipramine produced opposite changes on the different mRNA species assayed. The results reported here showed that CCK receptor gene expression was unaltered by the different drug treatments. In addition, chronic treatment with buspirone or imipramine did not affect the levels of either $GABA_B$ receptor or CCK transcripts. Alprazolam effects on CCK mRNA levels were consistent with previous studies reporting an effect of benzodiazepine site ligands on CCK gene expression. Acute diazepam treatment and withdrawal from chronic diazepam administration increase preproCCK mRNA levels in cortex and hippocampus (Rattray et al., 1993), whereas acute treatment with the benzodiazepine inverse agonist FG 7142 increases CCK transcript levels in the amygdala and CA_3 pyramidal cell layer of the hippocampus (Pratt and Brett, 1995). Hence, this is the first report of an effect of chronic benzodiazepine treatment on CCK mRNA levels. Evidence suggests that this increase in the amount of CCK transcript may correlate with an increase the level of the peptide, since chronic treatment (14 days) with two other antipanic agents, clonazepam (benzodiazepine) and clomipramine (tricyclic antidepressant), increased CCK-like immunoreactivity in the ventral tegmental area and in the cingulate cortex (Brodin et al., 1994). However, another group did not report any effect of chronic treatment with desipramine, imipramine, amitriptyline or citalopram on the various molecular forms of CCK in frontal cortex (Harro et al., 1997).

Another original finding from this study is that alprazolam also modulates $GABA_B$ receptor gene expression. The consequences and mechanisms of this increase in $GABA_B$ receptor and CCK transcript levels by alprazolam are somewhat difficult to anticipate. It is possible that alprazolam administration increases the number of post-synaptic $GABA_B$ receptors, which would enhance the late inhibitory phase mediated by synaptic GABA release. Alternatively, alprazolam treatment may trigger some feedback mechanisms onto the presynaptic neuron to elevate the presynaptic $GABA_B$ receptor population and decrease GABA release as a negative feedback effect. However at this stage, the alteration in $GABA_B$ receptor mRNA level observed cannot be attributed to either presynaptic or postsynaptic $GABA_B$ receptors, or to any $GABA_B$ receptor subtypes. Similarly, the increase in CCK mRNA level after chronic alprazolam treatment is unexpected, but it is possible that an increase in CCK levels may play a role in the development of tolerance to and/or withdrawal from benzodiazepine treatment. A number of animal studies have shown that anxious behaviors due to diazepam withdrawal, which can be blocked by CCK_B receptor antagonists (Woodruff et al., 1991; Wettstein et al., 1994; Harro et al., 1995) are concomitant with an increase in CCK mRNA and CCK_B receptor binding density in cortex and hippocampus (Rattray et al., 1993; Harro et al., 1995). The fact that chronic buspirone administration (which does not produce tolerance or withdrawal) had no effect on CCK, CCK_A and CCK_B receptors mRNA levels may support this view.

Finally, the finding that chronic treatment with phenelzine decreased CCK gene expression is novel, but the lack of effects the two other antipanic agents was unexpected. It is possible that a decrease in CCK levels induced by phenelzine may be part of the anxiolytic effect spectrum of this drug (Paslawski et al., 1996). The lack of any common effect amongst the antipanic agents on either CCK, $GABA_B$ or CCK receptors does not support the view of a common remodeling of the GABAergic/CCK transmission interaction by antipanic drugs. However, it is possible that the time frame selected is inappropriate. For clarity of the following discussion, I defined as time 0 (noted T) the time point that corresponds to the increase in $\alpha3$-, $\beta1$- and $\gamma2$-subunit gene expression for a particular drug. Relative to the changes in $\alpha3$-, $\beta1$- and $\gamma2$-subunit mRNA levels, the determination of the amount of CCK, $GABA_B$ or CCK receptor transcripts was performed at T+11 days for alprazolam but at T+0 day for the antidepressants. Thus, common changes in CCK, $GABA_B$ or CCK receptor mRNAs corresponding to a specific antipanic mechanism occurring in a defined time frame dependent on the onset of action of a particular agent may have been missed.

In summary, antipanic drugs shared effects on $GABA_A$ receptor $\alpha3\beta1\gamma2$ subunit gene expression, but seemed devoid of a common effect on $GABA_B$, CCK or CCK receptor gene expression. The time scale difference in the effect of alprazolam compared to the other two antipanic drugs may be linked to the earlier onset of action of this drug. The view that this common increase in $\alpha3\beta1\gamma2$ $GABA_A$ receptor subtype underlies the therapeutic onset of antipanic drugs is in concordance with both the neuroanatomical hypothesis of PD (Gorman et al., 1989) and the model of long-term pharmacological treatment of this disorder (Ballenger, 1991). Thus, the former hypothesis put the panic attack "center" as being in the brainstem, while the latter model proposes that the onset of antipanic therapy starts by a reduction in the number of symptoms. Hence, the first neuroanatomical location commonly targeted by antipanic agents may be the $GABA_A$ receptors in the brainstem.

7. General Discussion

Panic disorder is a common debilitating mental illness. Over the last 20 years, a significant number of studies have been conducted to uncover possible neurochemical abnormalities involved in PD. Research has focused mainly on the neurotransmitters noradrenaline (Charney et al., 1990a; Heninger, 1989), serotonin (Westenberg, 1996; Pecknold, 1990), GABA (Breslow et al., 1989) and CCK (Bradwejn, 1993b). While the etiology of PD was initially believed to involve only a single neurotransmitter system, current thinking has evolved to include interactions of multiple neurotransmitter systems (Zacharko et al., 1995). This latter approach stems from the fact that unique neurotransmitter dysregulation models cannot account for the fact that PD is induced in laboratory settings by chemical agents with diverse neurochemical and neuroanatomical targets (Papp et al., 1993; Gorman et al., 1987; Bradwejn, 1993b; Van Megen et al., 1996a), and that this illness is treated by numerous drugs with apparently different primary mechanisms of action (Johnson et al., 1995; Rosenberg, 1993; Bradwejn, 1993a; Ballenger, 1986; Westenberg, 1995; Curtis et al., 1993; Burrows et al., 1993; Layton and Dager, 1994; Ballenger, 1991; Westenberg, 1996). In addition, the onset of therapeutic action of the antidepressants with antipanic properties is delayed compared to their immediate effects on monoamine levels or inhibition of reuptake (Ballenger, 1991; Layton and Dager, 1994; Jefferson, 1997).

The latter observation suggests that neurotransmission remodeling likely underlies the belated onset of action of the antipanic agents. The choice for investigating GABAergic transmission arose from the fact that: (1) animal and clinical studies showed that GABAergic transmission blockade could trigger panic-like behaviors in animals and panic attacks in PD patients (Graeff et al., 1993; Graeff, 1994; Priolo et al., 1991; Nutt et al., 1990); (2) GABA is an ubiquitous CNS inhibitory transmitter with extensive interaction with many of the neurotransmitters potentially involved in PD (López-Rubalcava et al., 1992; Petty, 1995; Scatton et al., 1986; Suranyi-Cadotte et al., 1990; Zacharko et al., 1995); and (3) antipanic agents seem to commonly enhance GABAergic transmission (Baker et al., 1991; Korf and Venema, 1983; Paslawski et al., 1995; Dunn et al., 1994; Sieghart, 1995; Sethy and Harris, 1982; Sethy et al., 1983).

It is possible, therefore, that the therapeutic efficacy of antipanic agents results from common alteration(s) of GABAergic transmission. However, pre- or post-treatment plasma or CSF GABA levels in PD patients are not different from control levels (Roy-Byrne et al., 1992; Rimón et al., 1995), and GABA$_B$ receptor involvement in the etiology or pharmacotherapy of PD is controversial (Breslow et al., 1989; Engelbrecht et al., 1994;

McManus and Greenshaw, 1991). In contrast, there is evidence to suggest that there is an alteration of benzodiazepine receptor density and/or function in PD patients (Roy-Byrne et al., 1989; Roy-Byrne et al., 1990; Roy-Byrne et al., 1996; Cowley et al., 1995; Malizia et al., 1995; Kuikka et al., 1995; Kaschka et al., 1995; Schlegel et al., 1994), and studies have shown that chronic treatment with $GABA_A$ receptor ligands alter this receptor's binding characteristics and subunit gene expression (Calkin and Barnes, 1994; Heninger et al., 1990; Kang and Miller, 1991; Mhatre and Ticku, 1992b; Mhatre et al., 1993; Mhatre and Ticku, 1994a; Mhatre and Ticku, 1994b; O'Donovan et al., 1992; Primus and Gallager, 1992; Ticku and Mhatre, 1992; Tseng et al., 1993; Tseng et al., 1994). Consequently, the hypothesis that the common therapeutic effect of antipanic agents is due to a remodeling of $GABA_A$ receptor mediated transmission via alteration of the receptor subunit composition was tested. The choice of the multiprobe solution hybridization assay for studying this common alteration of $GABA_A$ receptor subunit gene expression by antipanic drugs was dictated by the fact that the subunit composition of all the existing $GABA_A$ receptor subtypes *in vivo* is not established. Further, the pharmacological tools available to distinguish between the different receptor subtypes are limited. Therefore, the identification and the quantification of the different $GABA_A$ receptor subtypes naturally present in the brain is difficult. Finally, there is evidence for a good correlation between the $GABA_A$ receptor subunit mRNA and protein levels (Fritschy and Mohler, 1995; Laurie et al., 1992; Wisden et al., 1992), and oligonucleotide probes that recognize specific mRNA molecules can be made readily.

7.1. MAJOR FINDINGS

The major findings presented in this dissertation fall into four categories. Firstly, an anxiolytic drug for which the primary mechanism of action does not involve $GABA_A$ receptors was able to modulate specific $GABA_A$ receptor subunit gene expression in a brain region-dependent manner. Buspirone increased α2-, γ1- and γ3-subunit gene expression in cortex but not in the cerebellum, and decreased α1-, β2- and β3-subunit gene expression in the brainstem. Secondly, antidepressant agents with different primary sites of action altered specific $GABA_A$ receptor subunit mRNA levels in a drug- and brain region-dependent manner. Phenelzine altered β1- and γ3-subunit transcript levels in the cortex, while in the cerebellum, imipramine increased α1-, α3- and β2-subunit mRNA levels. Thirdly, antipanic drugs commonly affected $GABA_A$ receptor subunit gene expression. In contrast to buspirone, the three antipanic agents tested, namely alprazolam, imipramine and phenelzine, increased cerebellar α6-subunit and brainstem

$\alpha3$-, $\beta1$- and $\gamma2$-subunit steady-state mRNA levels. Finally, the antipanic drugs did not seem to alter CCK system gene expression in the brainstem in a common manner. The three antipanic agents tested did not display any common effect on either CCK or $CCK_{A/B}$ receptor steady-state mRNA levels.

7.2. LIMITATIONS OF THESE STUDIES

7.2.1. Statistical analysis

The hypothesis tested is the null hypothesis which stipulates that there is no difference between the means of the groups tested, and the statistical analyses of the data were performed at the 5% confidence interval. Therefore, the probability of determining a significant difference by chance only (type I statistical error of false positive) is of 1 in 20. The small number of animals per group (n=6) is believed to have been a hindrance to the power of the statistical analysis. Indeed, variance and standard deviation values depend on the number of subjects per group. Further, for some subunit RNA levels, outliers could not be excluded from group data since these values lie within 2 standard deviations to the mean of the group. As a result, the variances of these treatment groups were markedly higher than those of the other groups, and this may have impeded the value of the mean square error for Dunnett's test. This higher mean square error likely reduced the probability of determining significant changes; therefore some type II statistical errors (false negative) may have occurred for the sets of data that included outliers.

7.2.2. Methodological limitations

The issue of not screening animals behaviorally before inclusion in these studies may raise the concern that individual variation in basal anxiety may have influenced the outcome of these experiments. It is indeed conceivable that inter-individual variation in anxiety state may exist in rats. These differences in basal anxiety levels probably reflect the differences in anxiety state that are naturally prevalent in the general population. These variations in anxious state possibly relate to differences in neurotransmission tone which in turn may relate to differences in neurotransmitter level and/or receptor function. Differences in the GABAergic system between subject could account for such a phenomenon, so that some individuals could present altered GABA levels and/or receptor subunit gene expression. Thus, these differences in anxious state among animals probably contribute to increase the group heterogeneity in the $GABA_A$ receptor subunit gene expression at baseline, and after drug treatment. However, the fact that changes in mRNA levels were found after drug administration suggests that the drug effect on gene expression overrides the natural heterogeneity. Thus, the selection of the animals according to their background anxiety levels may have reduced the groups variances and resulted in a better clear-cut effect of drug administration.

As mentioned in Chapter 3 and 5 discussions, an inter-experiment variation was associated with the solution hybridization assay used for the determination of the different mRNA levels. Although the source of this inter-replicate variation is unknown, this phenomenon was consistently observed for all the $GABA_A$ receptor subunit mRNA levels measured. Indeed, in the brainstem the third quartile for the inter-experiment variation for all the subunits was around 40%. This percentage variation indicates that the mean of 3 out of 4 experimental replicates are expected to lie within the range pooled mean ± 40% of the pooled mean value. Although the inter-experiment variation seems to remain constant for all the subunit mRNA levels measured, this inter-replicate variation appears to be conversely proportional to the abundance of the mRNA species. Indeed, as mentioned above, the third quartile inter-experiment variation on the measurement of the $GABA_A$ receptor subunit mRNA levels, that are 10-25 time less abundant than β-actin mRNA, is 40%. In contrast, the third quartile inter-replicate variation on the measurement of the GAD, GAT and GABA-T mRNA levels, that are almost as abundant as β-actin mRNA, is of 15%. Hence, this inter-experiment variation constituted a non-negligible technical limitation to the accurate determination of the mRNA levels.

The choice of studying the changes in $GABA_A$ receptor subunit gene expression after 21 days of treatment with antipanic agents was based on the clinical report of onset of improvement of PD patients after about 3-4 weeks of treatment (Westenberg, 1996; Layton and Dager, 1994; Liebowitz et al., 1986; Ballenger et al., 1988; Schweizer et al., 1993; Black et al., 1993). According to the hypothesis tested, the remodeling of the GABAergic transmission underlying the onset of antipanic therapeutic effects is exerted via variations in protein levels. Hence the changes in the corresponding mRNA amounts are likely to precede protein level variations and the onset of antipanic therapy, and thus may occur after 21 days of treatment. Clinical evidence suggests that the onset of antipanic efficacy may occur at an earlier time point for the benzodiazepines compared to the antidepressants (Ballenger, 1991; Bradwejn, 1993a), which, according to the Chapter 6 data discussed below, appears to take place after short-term alprazolam administration. Thus, the 21 day time point may be inappropriate for the study of the changes in $GABA_A$ receptor subunit gene expression underlying the onset of action of alprazolam. Consequently, it appears that with regard to alterations in $GABA_A$ receptor subunit mRNA levels, 21 days of treatment with imipramine or phenelzine parallels 3 days of treatment with alprazolam. Hence, there would be a time lag of about 18 days between the molecular events triggered by alprazolam and those induced by phenelzine and imipramine. Therefore, the changes in gene expression seen after 21 days of treatment with alprazolam might be similar to that which might be expected after 39 days of treatment with the antidepressants.

A similar argument regarding possible changes in $GABA_B$ and CCK system gene expression has been developed in Chapter 6. Defining as time 0, the time point at which alprazolam and the antidepressants increase α3-, β1-, and γ2-subunit gene expression, respectively, the time points investigated corresponded to 11 days after the changes in $GABA_A$ receptor α3-, β1-, and γ2-subunits mRNA levels for alprazolam, but that of time 0 for imipramine and phenelzine. Although phenelzine significantly decreased CCK transcript levels at 21 days of treatment (time 0), it is unlikely that these changes will be seen at the similar time point (time 0) for all the antipanic drugs tested since the imipramine induced no variations on either CCK system or $GABA_B$ receptor gene expression. In contrast, a complementary study after 32 days of treatment with phenelzine or imipramine (11 days after the changes in brainstem subunit gene expression) may be warranted since treatment for 14 days with alprazolam (11 days after the effects of alprazolam on brainstem subunits mRNA levels) induced significant changes in CCK and $GABA_B$ mRNA levels. Thus, the changes in the amount of $GABA_B$ receptor and CCK system transcripts seems to occur on a different time scale compared to that of $GABA_A$ receptor subunit mRNA levels. The time course of the alterations in gene expression appears to depend on both the drug and the system studied. Finally, as discussed below, different molecular events may underlie the different phases of therapeutic effects. Thus, the 21 day time point for alprazolam treatment may be suitable for the determination of possible molecular targets for subsequent therapeutic phases.

The molecular studies presented have some limitations in that the consequences of the observed alterations in the amount of subunit transcripts remain to be established. Hence, these changes in $GABA_A$ receptor subunit mRNA levels are expected to precede similar changes in the levels of the corresponding subunit proteins which would be part of a plasma membrane receptor. The confirmation that these changes in mRNA levels precede alterations in the amounts of the corresponding proteins, and the determination of the location of such $GABA_A$ receptor subunit composition remodeling would allow the extrapolation of the functional properties of these new receptors. In addition, the physiological relevance of these changes in $GABA_A$ receptor mediated-transmission at the brain nuclei level could be examined. The functional relevance of these changes in GABA$_A$ receptor subtypes could then be tested by electrophysiological and/or autoradiographic ligand binding experiments in the appropriate brain area.

Finally, these changes in GABA$_A$ receptor subunit gene expression were examined at the gross brain structure level, i.e. cortex, cerebellum and brainstem. Hence, the variations observed represent the average changes amongst all the nuclei and cell types present within the anatomical region investigated. Many localized or cell-specific changes have probably been missed as they were averaged with the alterations occurring in different areas.

7.3. POSSIBLE MECHANISMS OF ACTION FOR THE DIFFERENT DRUGS TESTED

While keeping in mind the limitations inherent to this study, the results suggest models for the mechanisms of action of the different drugs tested. In the cortex, long-term treatment with the anxiolytic agents alprazolam and buspirone seemed to particularly affect the level of expression of the $\alpha2\alpha4\beta1\gamma1$ GABA$_A$ receptor subunit gene cluster. The scatter of the upward regulation in the $\alpha2\alpha4\beta1\gamma1$ gene cluster mRNA levels induced by buspirone was markedly greater than that produced by alprazolam. This up-regulation in $\alpha2\alpha4\beta1\gamma1$ gene cluster expression seems to be shared with other anxiolytic drugs (such as diazepam) in a time frame, 7 to 14 days of treatment (Holt et al., 1996), which corresponds to the maintenance of their anxiolytic effect, before the development of tolerance. The anxiolytic effect of buspirone is characterized by a delayed onset requiring chronic administration, but long-term administration of this agent does not produce tolerance or withdrawal. The confirmation that the effects of buspirone on the $\alpha2\alpha4\beta1\gamma1$ gene cluster occur and are sustained under chronic treatment only, whereas the effects of alprazolam are induced in the short-term would support the view that an increase in $\alpha2\alpha4\beta1\gamma1$ gene expression in the cortex may contribute to the anxiolytic effects of these drugs rather than their adverse effects. The functional consequence of an increase in $\alpha2$-subunit gene expression is of special interest in light of recent immunohistochemical studies showing that the $\alpha2$ subunit is mostly present on the axon initial segment of layer II-III pyramidal cells (Fritschy et al., 1998). Pyramidal cells are thought to be important in the regulation of the somatosensory inputs to the cortex (Berman et al., 1992; Ebner and Armstrong-James, 1990), and their axon initial segment is innervated by GABAergic chandelier cells (Berman et al., 1992; Barnstable et al., 1992). Indeed, in the pyriform cortex, layer II-III pyramidal cells receive a convergence of inputs from GABAergic interneurons activated by various monoamines (Gellman and Aghajanian, 1993). Thus, the chandelier neuron input on the initial segment of pyramidal cells is an important regulator of the pyramidal cell firing rate (Berman et al., 1992; Barnstable et al., 1992). An increase in the number of $\alpha2$-subunit containing $GABA_A$ receptors triggered by anxiolytic agents may alter the chandelier cell GABAergic gating of the processing of the converging inputs to the pyramidal cells.

The mechanisms by which phenelzine and imipramine or any other drug may alter $GABA_A$ receptor subunit gene expression is not currently defined. However, evidence indicates that the spectrum of action of these drugs may involve gene regulation, and in particular, the regulation of the gene expression of neurotransmitter system proteins that include synthesizing enzymes, transporters or receptors. Chronic imipramine treatment decreases the level of tyrosine hydroxylase mRNA levels and protein activity in the ventral tegmental area (Lavergne et al., 1994), whereas chronic desipramine increases glucocorticoid receptor mRNA and protein levels, as well as the glucocorticoid receptor gene promoter activity (Barden et al., 1995). In other studies, chronic desipramine blocked reserpine-induced increases in tyrosine hydroxylase mRNA in the locus coeruleus (Schultzberg et al., 1991), and chronic administration of desipramine or trancylpromine (a MAO inhibitor) altered brain-derived neurotrophin factor and its receptor, trkB, mRNA levels in hippocampus (Nibuya et al., 1995). Finally, chronic administration of antidepressants that block serotonin reuptake, but not serotonin metabolism, decreased serotonin transporter gene expression in the raphé (Lesch et al., 1993). In contrast, a few studies did not report any effects of antidepressants on gene expression: chronic antidepressant treatment does not affect $5\text{-HT}_{1A/1B/1C/2}$ receptors, tryptophan hydroxylase, aromatic amino acid decarboxylase (Spurlock et al., 1994), serotonin transporter (Spurlock et al., 1994; Linnet et al., 1995) or glutamate receptor (Oretti et al., 1994) mRNA levels. However, these studies were conducted in whole brain homogenates and may have failed to reveal any regional alterations in gene expression.

Further evidence supports the view that antidepressants also influence neuronal activity. Acute treatment with antidepressants upregulated Fos, an immediate-early gene product which is a marker of acute cellular activity, mRNA and protein levels (Beck, 1995). However, Fos also acts as a transcription factor (Sheng and Greenberg, 1990). Therefore, the fact that chronic antidepressant administration specifically alters the mRNA and protein levels of Fos throughout the brain (Beck and Fibiger, 1995; Morinobu et al., 1995) suggests that the long-term effects of these drugs involve the regulation of the expression of specific genes. In addition, imipramine and other tricyclic antidepressants have also been shown to inhibit cAMP response element-binding protein (another transcription factor) and cAMP response element-directed gene transcription (Schwaninger et al., 1995). Further, the antidepressant mianserin alters 5-HT$_2$ receptor gene expression by modulating the receptor transcription at the promoter level (Toth and Shenk, 1994), but chronic desipramine and fluoxetine have differential effects on 5-HT$_2$ receptor-mediated *c-fos* gene activation (Tilakaratne et al., 1995). These latter results suggest that antidepressant drugs may differentially interfere with the transcription machinery. Hence, the cascade of events triggered by the antidepressants, leading to the modulation of GABA$_A$ receptor subunit gene expression, may involve the cAMP response element pathway and/or immediate-early genes such as *c-fos*.

In eukaryotic cells, gene transcription involves numerous steps and proteins. Briefly, transcription factors bind to the gene promoter to facilitate anchoring of the RNA polymerase complex at and over the transcription start site, and regulate the rate of transcription (Sheng and Greenberg, 1990; Lemke, 1992). Some transcription factors like Fos dimerize in order to bind to specific DNA sequences located in the promoter region of the gene to be transcribed (Sheng and Greenberg, 1990; Lemke, 1992; Hughes and Dragunow, 1995). Fos interacts with another protein, Jun, to form a heterodimeric transcription factor called activating protein-1 (AP-1) (Sheng and Greenberg, 1990; Lemke, 1992; Hughes and Dragunow, 1995). Depending on which Fos and Jun families members are forming the protein dimer, the increase in gene expression can vary from none to many orders of magnitude (Hughes and Dragunow, 1995). Evidence suggests that GABA$_A$ receptor subunit genes are also probably regulated by both cAMP and Fos pathways. For example, an early study has reported that the AP-1 transcription factor binds to the human α1-subunit gene promoter (Kang et al., 1994). Other investigations have reported that the 5' region of the human β3-subunit gene can bind AP-1 and CREB transcription factors (among others such as TFIID, OCT1, Sp1, CTF/NF1, AP-2 and GRE) (Kirkness and Fraser, 1993), and that the 5' region of the human α5-subunit gene displays consensus binding sequences for these transcription factors (Kim et al., 1997). Further, exposure of cerebellar granule cells in culture to agents that increase intracellular cAMP levels induces bi-directional changes in GABA$_A$ receptor α1- and α6-subunit mRNA levels (Thompson et al., 1996). The increase in α1-subunit transcript amount is matched by a increase in the number and affinity of diazepam sensitive binding sites, whereas there is no alteration in the characteristics of the diazepam insensitive binding sites to accompany the decrease in α6-subunit mRNA levels (Thompson et al., 1996). These results indicate that the regulation of GABA$_A$ receptor subunit gene expression by intracellular pathways is specific, and that changes in subunit transcript levels can be accompanied by parallel alterations in the amount of the corresponding protein.

Throughout the brain regions studied, the spectrum of alterations in $GABA_A$ receptor subunit gene expression induced by phenelzine overlaps but does not completely match with that of imipramine. Hence, the cellular mechanisms activated by imipramine do probably differ somewhat from those activated by phenelzine. Indeed, part of the effects of phenelzine may be due to the observed rise in brain GABA levels (Baker et al., 1991; Paslawski et al., 1995) which may be mediated by an increase in extracellular GABA (Parent et al., 1998). In contrast, the intracellular pathway recruited by imipramine remains unclear, especially since its primary active metabolite, desipramine, may account for some of the effects produced by imipramine treatment. Imipramine and desipramine differ in their pharmacological profiles: imipramine preferentially inhibits serotonin reuptake, whereas desipramine is a more potent noradrenaline reuptake inhibitor. Therefore, some changes in $GABA_A$ receptor subunit gene expression induced by imipramine may relate to serotonin reuptake inhibition whereas others may be linked to noradrenaline reuptake inhibition. Hence, the study of the effects of chronic treatment with selective noradrenaline or selective serotonin reuptake inhibitors on $GABA_A$ receptor subunit mRNA levels may allow the determination of which changes produced after imipramine treatment can be attributed to the inhibition of either noradrenaline or serotonin reuptake.

The results presented in this dissertation also suggest that the brainstem and cerebellum may be key neuroanatomical structures for the therapeutic effects of antipanic agents. In the brainstem, alprazolam, imipramine and phenelzine altered α3-, β1- and γ2-subunit gene expression. As mentioned previously, the time scale of this effect on $GABA_A$ receptor subunit gene expression differs among the drugs. In addition, this effect seems transitory, at least for alprazolam, and appears to reflect the differences in the relative onset of therapeutic action of these drugs. Indeed, the time lag of 18 days between the molecular effects produced by alprazolam and the antidepressants seems to reflect the gap between their respective onset of therapeutic action, suggesting that this increase in α3-, β1- and γ2-subunit transcript level may be a molecular basis for the onset of antipanic effect (Figure 7.1). As such, it is speculated that, although shifted in time by about 18 days, the alterations in α3-, β1- and γ2-subunit mRNA levels induced by imipramine and phenelzine should, as for alprazolam, be transitory and of similar time span (Figure 7.1). In order to confirm the above hypothesis, a time study of the transient nature and the duration of this modulation in α3-, β1- and γ2-subunit mRNA levels in the brainstem, should reveal a different occurrence time, but a similar duration, of this effect for the different drugs.

The antipanic drugs also affected the expression of the α6-subunit gene in the cerebellum. Here, α6-subunit gene expression is normally restricted to the granule cell layer (Laurie et al., 1992; Persohn et al., 1992) where the major $GABA_A$ receptor subtypes are thought to be composed of the following subunit combinations: α1β2/3γ2, α6β2/3γ2, α6α1β2/3γ2, α6α1β2/3δ and α6β2/3δ (Wisden et al., 1996; Jechlinger et al., 1998). Taking into account the relative abundance of the different mRNA species in the cerebellum, the increasing effect of antipanic drugs on α6-subunit transcript levels was accompanied by a similar effect on β2- and β3-subunit mRNA levels. In addition, there was no matching alteration in α1-subunit transcript level, suggesting that the population of α1-subunit containing $GABA_A$ receptors (i.e. α1β2/3γ2, α6α1β2/3γ2 and α6α1β2/3δ) remains unchanged. Further, in contrast to buspirone, the antipanic agents did not produce a matching up-regulation of γ2-subunit gene expression, suggesting that α6β2/3γ2 $GABA_A$ receptor subtype is unlikely to be the receptor subunit combination gene expression commonly affected by antipanic drugs. Hence, it is possible that this increase in α6-subunit probably reflects an augmentation in the number of α6β2/3δ $GABA_A$ receptors. The distribution of the δ subunit is also restricted to the granule cell layer in the cerebellum (Persohn et al., 1992; Laurie et al., 1992; Fritschy and Mohler, 1995). A recent study reported that inactivation of the α6-subunit gene was accompanied by a selective post-translational degradation of the δ subunit in cerebellar granule cells, suggesting a specific association between the α6 and δ subunits in the cerebellum (Jones et al., 1997). Therefore, it is possible that the increase in α6-subunit containing receptors observed after chronic treatment with antipanic agents corresponds to an up-regulation in the α6β2/3δ receptor subunit combination.

The confirmation that the amount of δ-subunit mRNA is increased by antipanic drugs would be of special interest in the light of recent studies showing that the distribution of cerebellar $GABA_A$ receptor subtypes is segregated (Nusser and Somogyi, 1997; Nusser et al., 1996). Hence, α6β2/3δ $GABA_A$ receptor subtypes appear to be present exclusively on extrasynaptic membranes of cerebellar granule cells (Nusser et al., 1996; Nusser et al., 1998). Functional studies have shown that the α6β2/3δ $GABA_A$ receptor subtype displays a high affinity for GABA (Saxena and Macdonald, 1996), that δ-subunit containing receptors do not desensitize upon an extended exposure to an agonist (Saxena and Macdonald, 1994), and that $GABA_A$ receptors of granule cells could mediate both phasic and tonic responses (Wall and Usowicz, 1997; Brickley et al., 1996).

Hence, the phasic inhibition may be mediated by synaptic GABA$_A$ receptors, while the tonic inhibition results from the activation of extrasynaptic receptors (Wall and Usowicz, 1997; Brickley et al., 1996), such as the $\alpha6\beta2/3\delta$ receptor subtypes (Nusser et al., 1998). Therefore, the increase in $\alpha6$-subunit gene expression induced by the antipanic drugs may be related to an enhanced GABAergic tonic inhibition mediated by an increase in the number of non-synaptic $\alpha6\beta2/3\delta$ GABA$_A$ receptor subtype present on cerebellar granule cells. This enhanced tonic inhibition on granule cells would reduce their activation of Purkinje cells which in turn may lead to a decreased Purkinje cell inhibitory output to the deep cerebellar nuclei or other brain centers. The consequences of a decreased cerebellar cortex inhibitory output are difficult to consider beyond the cerebellum. Indeed, the cerebellar cortex projects to the deep cerebellar nuclei (which contain inhibitory neurons) and to brainstem or limbic structures. Therefore, depending on the Purkinje cells that are functionally affected, the end result can be an enhanced inhibition (by relief of the inhibition of the cerebellar nuclei neurons, which in turn inhibit more strongly the structures to which they project) or an enhanced activation.

Evidence suggests that cerebellar function may be abnormal in anxiety disorders and PD in particular. Two PET studies have reported cerebellar activation during chemically-induced panic attacks in PD patients (Reiman et al., 1989; Benkelfat et al., 1995) and anxiety attacks in post-traumatic stress disorder patients (Brannan et al., 1997; Bremner et al., 1997). It is now well established that the cerebellum plays a role in the control of saccadic eye movement (Noda, 1991). Therefore, the reduced effect of diazepam on saccadic eye movement described in anxious patients (Roy-Byrne et al., 1989; Roy-Byrne et al., 1990; Roy-Byrne et al., 1996) may be indicative of a decreased number of benzodiazepine-sensitive sites in the cerebellum of anxiety disorder patients. Results from imaging studies investigating benzodiazepine receptor density with ^{123}I-iomazenil reported no alteration in the cerebellum of PD patients compared to matched healthy volunteers (Kuikka et al., 1995). However, this radioligand does not distinguish between the different $\alpha\beta\gamma2$ subunit combinations (Johnson et al., 1990; Wisden et al., 1996), so any variation in a specific receptor subtype may be missed. Studies have shown that the $\alpha1$ subunit is expressed by basket, Purkinje and granule cells (Wisden et al., 1996). On the last of these, $\alpha1$-subunit containing receptors have been located at the GABAergic Golgi synapses, as well as at extrasynaptic membrane (Nusser et al., 1996; Jones et al., 1997; Nusser et al., 1998; Nusser and Somogyi, 1997). Thus, a decrease in $\alpha1\beta\gamma2$ receptors in the cerebellum may be responsible for both a decreased sensitivity to benzodiazepines in anxious patients, and an enhanced cerebellar activation during anxiety episodes. Consequently, an increase in α6β2/3δ receptors produced by antipanic agents may restore the cerebellar inhibitory function.

It is striking that the pharmacotherapy of PD has been broken into acute, stabilization and maintenance phases (Ballenger, 1991) which parallel the alleviation of different sets of symptoms (panic attacks, anticipatory anxiety and phobic avoidance behavior) (Rosenberg, 1993), and are postulated to involve different brain regions (brainstem, limbic lobe and prefrontal cortex, respectively) (Gorman et al., 1989). Therefore, it is possible that distinct regional molecular events may underlie these different therapeutic phases. While the increase in α3-, β1- and γ2-subunit gene expression may underlie the onset of therapeutic action of the antipanic agents, an augmentation in the number of α6β2/3δ receptors in the cerebellar granule cells may be linked to the therapeutic stabilization phase (Figure 7.1). Thus, this speculation adds a new brain area to the neuroanatomical model proposed (Gorman et al., 1989) by suggesting that molecular events in the cerebellum are involved in the relief of anxiety that corresponds to the therapeutic stabilization phase (Figure 7.2). Finally, as mentioned above, this hypothesis is also consistent with the findings of cerebellar activation during anxiety episodes (Reiman et al., 1989; Benkelfat et al., 1995; Brannan et al., 1997; Bremner et al., 1997).

Such a view would explain why the effects of alprazolam on α3-, β1- and γ2-subunit gene expression are transient and occur after subchronic treatment in the brainstem, whereas the increase in α6-subunit mRNA in the cerebellum after alprazolam treatment presents an amplitude similar to that induced by imipramine and phenelzine and is seen after chronic administration, at a time point which corresponds to phenelzine's and imipramine's onset of therapeutic effect. As illustrated in Figure 7.1, this hypothesis implies that the increase in cerebellar α6 subunit reaches its maximum amplitude during the onset of the therapeutic effect, and is maintained well after the decline of this phase. Evidence suggests that such a pattern of modulation of α6-subunit mRNA levels by chronic drug treatment is possible. Indeed, O'Donovan et al. (1992) have described a 23-50% (95% confidence interval) induction of α6 subunit gene expression in whole brain over the course of 32 days of flunitrazepam administration. After 4 days of flunitrazepam delivery, the levels of α6-subunit mRNA rise slightly (O'Donovan et al., 1992). However, after 7 days of treatment, the change in α6-subunit transcript levels becomes significant and then remains elevated for the duration of the treatment (O'Donovan et al., 1992). Since α6-subunit gene is mainly expressed in the cerebellum (Laurie et al., 1992; Fritschy and Mohler, 1995), this delayed and sustained effect of flunitrazepam on α6-subunit mRNA levels is likely to relate to a cerebellar effect.

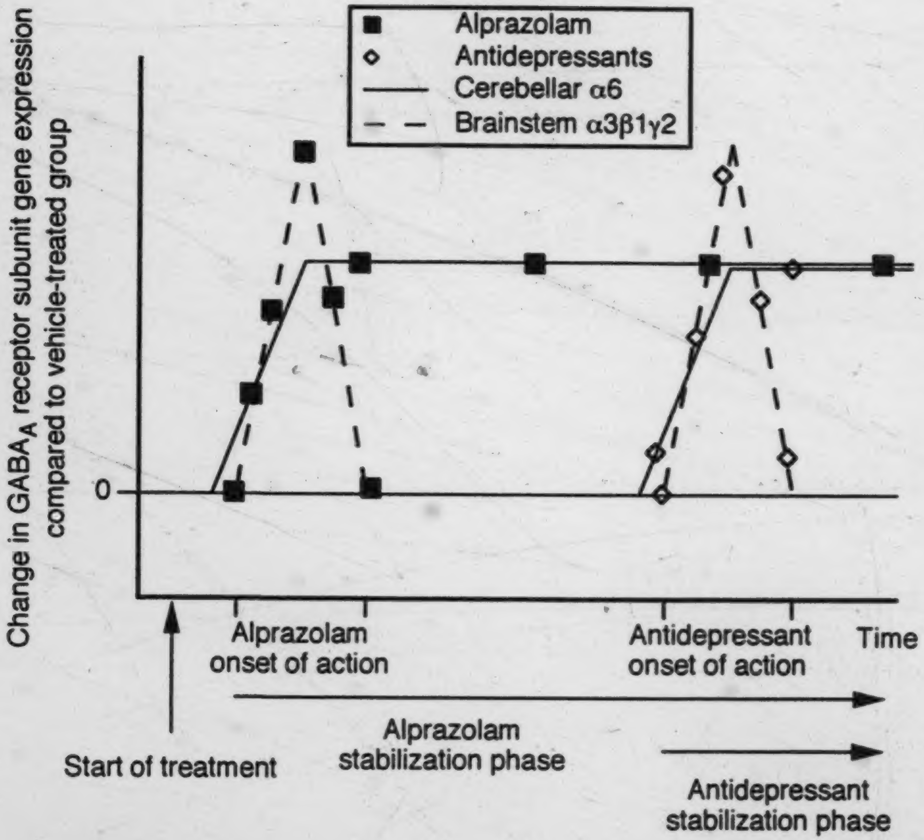

Figure 7.1 Schematic diagram of the molecular events hypothesized to be involved in the different therapeutic phases of PD treatment
This diagram illustrates the hypothesized relationship between the changes in cerebellar α6- (plain line) and brainstem α3-, β1- and γ2-subunit (dashed line) gene expression and the onset and stabilization of the therapeutic effects.

To test this hypothesis one would have to determine the levels of α6-, β2-, β3- and δ-subunit mRNAs after administration of the various drugs for 3 or 39 days. In order to validate this hypothesis, short-term administration of alprazolam should increase α6-, β2-, β3- and δ-subunit gene expression, whereas after 39 days of treatment (18 days after the effect of phenelzine and imipramine on brainstem subunit gene expression) at least the two other antipanic agents shall maintain their upward regulating effects on these mRNA levels. Finally, the hypothesis that common molecular mechanisms occurring earlier in time for the benzodiazepines underlie the various treatment phases may explain the rationale behind the clinical practice of a co-administration of a benzodiazepine and an antidepressant. Such a drug combination takes advantage of the earlier therapeutic onset of the benzodiazepine, which is tapered off without the recurrence of panic attacks after about 3 weeks of treatment (once the stabilization phase has been reached for both agents).

7.4. CONCLUSION

The results from the studies reported in this dissertation are a first step toward the elucidation of the mechanisms of action of antipanic agents. However, these results are not predictive of the neurobiology of PD. The negative results of the study of common effects of the antipanic agents on $GABA_B$ receptor and CCK transmission parameters are not in favor of a CCK system and/or GABA/CCK transmission interaction mediating the pharmacotherapeutic effects of all antipanic agents. However, as mentioned above, it is possible that the time points investigated were inadequate, and/or that these neurotransmitter system interactions may be more relevant to the etiology of PD rather than the pharmacotherapy of this disorder.

The hypothesis tested in this dissertation of a common effect of antipanic agents on $GABA_A$ receptor subunit gene expression has been validated. Antipanic drugs do affect a specific set of $GABA_A$ receptor subunit mRNA levels in the brainstem and cerebellum. The neuroanatomical hypothesis for PD developed by Gorman et al. (1989) could be combined with the observed stepwise therapy of PD (Ballenger, 1991; Rosenberg, 1993) to generate a neuroanatomical pharmacotherapeutic model of PD (Figure 7.2). According to this new model, the neurotransmission remodeling in the brainstem underlies the alleviation of panic attacks, correlating with the onset of antipanic therapy, whereas the maintenance of the therapeutic effect related to the relief of anxiety is triggered by molecular events in the cerebellum. The neuroanatomical pathways possibly involved in this pharmacotherapeutic model are not defined at present. However, the numerous pathways that exist between these different brain regions are of prime interest. The projections between the brainstem nuclei such as the LC or the pontine nuclei and the cerebellum and cortex are well documented (Olson and Fuxe, 1971; Mason and Fibiger, 1979; Brodal and Bjaalie, 1992; Dietrichs et al., 1994). The nucleocortical and the corticonuclear cerebellar projections as well as the direct and indirect hypothalamocerebellar and cerebellohypothalamic pathways have been mapped (Batini et al., 1989; Batini et al., 1992; Wojtowicz et al., 1978; Mouginot and Gahwiler, 1995; Dietrichs and Haines, 1989; Dietrichs et al., 1994). Consequently, the molecular events observed in the present studies, in the brainstem and cerebellum, may themselves be linked to the occurrence of further neurotransmission remodeling in other brain areas such as the limbic lobe and prefrontal cortex argued to be also involved in the anticipatory anxiety and phobic avoidance symptoms, respectively (Gorman et al., 1989). Therefore, the determination of the regional distribution of the alterations in $GABA_A$ receptor gene expression combined with the knowledge of the topographical organization of the neuronal circuitry within the identified brain structure(s) will provide insight into the afferent or efferent pathways and brain nuclei possibly involved in the pharmacotherapeutic effect of antipanic agents.

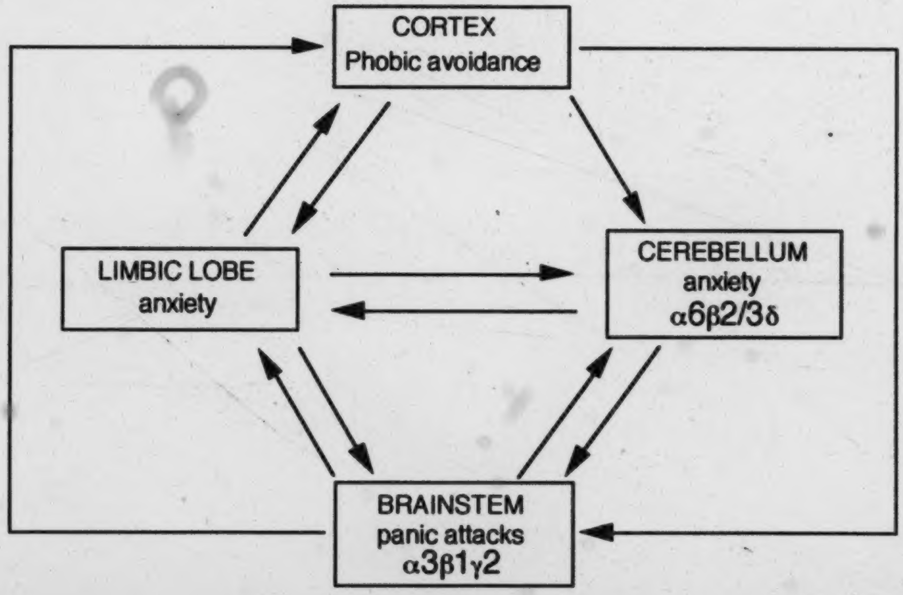

Figure 7.2 Schematic diagram of the neuroanatomical distribution of the molecular events speculated to alleviate the symptoms of PD and the main pathways relating these different areas

7.5. FUTURE WORK

Future work to address the functional significance of these changes in $GABA_A$ receptor subunit gene expression and to uncover the cellular mechanisms underlying the effects of antipanic agents, can be divided along two main research axes. On one side, the functional consequences of the alteration in $GABA_A$ receptor subunit mRNA levels and the link between the occurrence of these molecular events and the therapeutic effects of antipanic agents need to be examined. The neuroanatomical location and the functional consequences of the observed alterations in $GABA_A$ receptor subunit mRNA levels at the brain region and cellular levels ought to be determined. Thus, *in situ* hybridization experiments should identify the brain cells and nuclei where these molecular events take place, whereas immunohistochemistry, radioligand autoradiography and electrophysiological studies should determine the functional consequences of these changes in gene expression. In addition, these studies shall also indicate which neurons and transmission pathways bear these changes in gene expression. Consequently, the knowledge of the neuroanatomical projections of these neurons should identify other brain structures that will be susceptible to bear further molecular events. In parallel, the time point studies of these alterations in $GABA_A$ receptor subunit mRNA levels shall establish the relationship between these changes in gene expression and the therapeutic phases of PD treatment.

On the other hand, the intracellular pathways involved in these specific changes in gene expression need to be elucidated. For example, the effects of imipramine on gene expression can be attributed to either the parent drug or its primary active metabolite, desipramine, which differ in their pharmacological profiles. These molecules are distinguished by their differential affinity for serotonin and noradrenaline transporters. By comparing the alterations in gene expression produced by chronic treatment with either a specific noradrenaline or serotonin reuptake inhibitor (e.g., maprotiline and sertaline, respectively), it may be possible to attribute the effects of imipramine on gene expression to a specific pharmacological effect. Further, such a comparison may allow one to link the alterations in gene expression shared with the other antipanic agents used in the present studies to either serotonin or noradrenaline reuptake inhibition. Another way to investigate the cellular pathways involved in the regulation of the $GABA_A$ receptor subunit gene expression is to ascertain the effects of drug treatment on the transcription machinery. For example, the measurement of the changes over time in the amount of protein available to bind to a transcription factor DNA consensus sequence upon drug treatment may identify transcription factors and point toward intracellular pathways regulating the transcription of $GABA_A$ receptor genes. Finally, the elucidation of the cellular and anatomical pathway involved in the antipanic effect of drugs can be expected to lead to the rational development of new therapeutic agents with improved efficacy and reduced adverse effects.

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